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Constitutive expression of Jagged-1 in 3T3-L1 preadipocytes suppresses adipogenesis and adipose tissue development

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CONSTITUTIVE EXPRESSION OF JAGGED-1 IN 3T3-L1
PREADIPOCYTES
SUPPRESSES ADIPOGENESIS AND ADIPOSE TISSUE DEVELOPMENT

BY

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BA, Colby-Sawyer College, 2004

THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the
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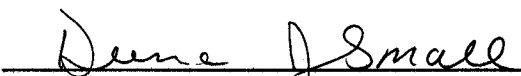


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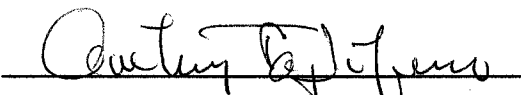
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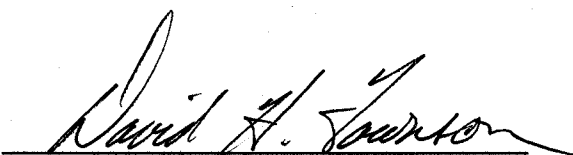
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DEDICATION

This thesis is dedicated to A. Orris Allen.

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I would like to thank my parents and Marc for their constant support and encouragement during my graduate school career. I would also like to thank my brothers Matthew and Shaughn.

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**CONSTITUTIVELY EXPRESSED JAGGED-1 IN PREADIPOCYTES
INHIBITS ADIPOGENESIS *IN VITRO* AND *IN VIVO*.**

By

Jennifer Allen

University of New Hampshire, December, 2007

The Notch signaling pathway regulates adipogenesis. To determine specific effects the Notch ligand Jagged-1 has on the regulation of adipogenesis, we transfected 3T3-L1 preadipocytes with either a constitutively expressed full-length human Jagged-1 construct or an empty vector control. These cells were grown in conditions promoting differentiation and then evaluated for lipid accumulation and gene expression. To test the role of Jagged-1 in adipose tissue development *in vivo*, transfected cells were examined for their ability to form fat pads after injection into nude mice. *In vitro*, cells constitutively expressing Jagged-1 accumulated less lipid and exhibited a delay in adipocyte protein expression. *In vivo*, mice injected with Jagged-1 transfected cells developed smaller fat pads than mice injected with L1 control cells. Thus, increased expression of Jagged-1 in L1 preadipocytes impaired adipogenesis as determined by changes in lipid uptake, expression of adipocyte-specific genes, and formation of fat pads in nude mice.

CHAPTER I

LITERATURE REVIEW

Obesity has become a leading cause of morbidity and mortality in the United States. Obesity-related health issues make up 6.8% of US health care costs (Mokdad, 1999) and include complications such as type II Diabetes, high blood pressure, cardiovascular disease cerebrovascular disease, peripheral vascular disease and cancer. The development of obesity and obesity-related health issues is multi-factorial. The generation of fat cells (adipocytes) is dependent on an orchestration of a variety of signaling cascades modulated by an array of hormones, cytokines, and other factors (reviewed in Gregoire, 1998; Farmer, 2006). Understanding how these factors work together to regulate the development of new adipocytes (adipogenesis) will provide the knowledge necessary to develop treatments for obesity-related diseases.

The Notch signaling cascade is one mechanism that regulates adipogenesis. The Notch signaling cascade plays a role in cell-fate determination of a number of cell types (Leforta, 2004; Sciaudone, 2003; Small, 2003) and influences adipogenesis (Garces, 1997; Ross., 2004; Ross, 2006). However, the mechanism by which it controls adipogenesis is not completely elucidated. This literature review will discuss the current research on adipogenesis and the role of Notch activation in regulating adipogenesis.

Obesity is the Result of an Over-abundance of Adipose Tissue.

Adipocytes develop as the result of an increased need for lipid storage. Fat tissue can expand through hyperplasia, which is the process by which adipocytes multiply in response to an increased need for energy storage (reviewed in Spiegelman, 1996; Spiegelman, 2001). Adipocytes can also accommodate more lipid by increasing in size (hypertrophy). Both hypertrophic and hyperplastic growth mechanisms can increase adiposity in an individual.

The incidence of obesity is on the rise in the United States. A 1991 National Health and Nutrition Examination Survey (NHANES) study determined that 12% of the United States adult population is obese (Mokdad, 1999). Comparison of NHANES III to cross-section nationwide studies of previous years, determined that the average BMI of adolescences (6-17 years old) and adults (20-70 years old) has increased. For example, girls and boys between 12 and 14 years of age had an average increase in BMI of 7% (Flegal, 2000). Adults between 50 and 59 years of age had an increased obesity prevalence of 11.4% and 13.3% in men and women, respectively (Flegal, 2000). The increase incidences of obesity could be the result of environmental factors and/or an increase in the number of people with genetic predisposition.

Research supports a role for a variety of genetic factors in the promotion of obesity. Mutations in genes that regulate fat storage can cause energy imbalance disorders (Mutch, 2006). For example, mutations in the Leptin gene which regulate appetite, inhibits Leptin from being up regulated. Since Leptinn

tells the brain it is no longer hungry, inhibiting the up regulation of Leptin results in a person that can not be satiated (Farooqi, 2002).

Genetic predisposition can be exacerbated by environmental factors. Environmental factors include, but are not limited to, the reduction in physical labor resulting from technological advancement and/or the increased consumption of fast food. The effect of exercise on weight loss was studied in a randomized study of 120 overweight middle age men and women (Slentz, 2004). Weight loss was two fold greater in subjects that participated in an exercise routine than subjects that did not (Slentz, 2004). Low levels of physical activity and large consumption of calories increases incidence of obesity. Thus, genetics make some people more prone to obesity which can be exacerbated by certain environmental factors.

Obesity can have many negative effects on the body that result in a variety of disease states. Individuals that exhibit obese conditions commonly have a premature mortality rate. NHANES studies determined that mortality rates are greater in obese adults with 111,909 extra deaths compared to average weight adults (Flegal, 2005; Mokdad, 1999). Some health complications that accompany obesity include sleep apnea (Peppard, 2000), asthma (as reviewed in Shore, 2007), cardiovascular disease (reviewed in Safar, 2006), and type 2 Diabetes Mellitus (Mokdad, 2001). Behavior Risk Factor Surveillance System (BRFSS) questionnaires produced by the Center for Disease Control and Prevention and state health departments have found that adults with a body mass index (BMI) of 40 or greater had a greater chance (95% confidence interval) of being diagnosed

with type 2 Diabetes Mellitus, asthma, high blood pressure, and high cholesterol (Mokdad, 2003). Since increased adiposity can have detrimental health effects, old therapies need to be improved and new ones developed to treat the increasing number of individuals that are experiencing obesity related complications. In order to generate such therapies, mechanisms regulating the development and function of adipose tissue need to be better understood.

Understanding the Process of Adipose Tissue Development is Vital for the Creation of New Treatments for Obesity.

Adipogenesis is the process by which preadipocytes differentiate and develop into mature adipocytes (reviewed in Gregoire, 1998). Mature adipocytes can be identified by their lipid-filled cytoplasm and by the expression of adipocyte-specific proteins. Some of these proteins assist in the storage of lipid and others are secreted as hormones and cytokines that have physiological effects on adipose and other tissue. For example, fatty acid binding protein (FABP4/aP2) is a cytoplasmic transport protein that binds to fatty acids and carries them within the cell (Hotamisligil, 1996; Maeda, 2003). Glycerol-3-phosphate dehydrogenase (GPDH) is an enzyme involved in the production of NADPH in the pentose phosphate pathway and is expressed in adipocytes as they begin to accumulate lipid (Bhandari, 1991). Lipoprotein lipase (LPL) is another protein that promotes the storage of lipid. Stimulated by insulin, LPL promotes the lipolysis of triglycerides in the blood into free-fatty acids, allowing them to be taken up by adipocytes (Mead, 2002). Leptin is a protein secreted by adipocytes that regulates the development of new adipose tissue by relaying messages to the brain

to decrease food intake (Halaas, 1995). These and a variety of other proteins secreted by mature adipocytes can be used to identify mature cells in culture.

Pluripotent mesenchymal stem cells (MSC) are present in a variety of tissues (Campagnoli, 2001; Romanov, 2003). The fibroblast-like MSCs differentiate into different concomitant cell lineages when exposed to molecular messages or 'signals' that are released from other cells in adipose and surrounding tissues (Figure 1). These cell lineages include chondrocytes, osteoblasts, myoblasts, and fibroblasts (Gimble, 1996; Tontonoz, 1994). MSCs also develop into preadipocytes, which are fibroblast-like precursors of adipocytes (Dani, 1997). Preadipocytes remain dormant in adipose tissue so that when necessary, they can differentiate into lipid filled adipocytes accommodating the body's energy-storage needs. Therefore, the differentiation of MSCs into preadipocytes marks the beginning of adipogenesis.

Interactions between molecular signals and receptors present on the surface of preadipocytes promote the proliferation of preadipocytes (Figure 1). These signals are secreted into the bloodstream and the extracellular space by surrounding adipocytes and other cells. Signals promoting proliferation include hormones such as insulin and glucocorticoids, as well as cytokines such as Fibroblast Growth Factor (FGF) and Endothelial Growth Factor (EGF) (Adachi, 1994; Hutley, 2004). Preadipocytes proliferate and remain in adipose tissue until they receive further signals that cause some of them to exit the cell cycle and differentiate into adipocytes. *In vitro*, the addition of insulin to culture media promotes the proliferation of preadipocytes (Green and Kehinde, 1975). The

addition of glucocorticoids to insulin containing media further increases preadipocyte proliferation (Gregoire, 1991; Kras, 1999). Thus, the proliferation of preadipocytes is promoted by synergistic interactions among multiple factors.

Proliferation and differentiation of preadipocytes can be seen during times of growth such as fetal development, infancy, and puberty. In addition to stimulating the proliferation response, insulin and glucocorticoids also promotes differentiation by increasing the transcription of adipocyte-specific genes (Green and Kehinde, 1975; Gregoire, 1991; Ross, 1999). The addition of these two hormones to culture media enhances the expression of adipocyte-specific markers such as LPL and GPDH, and lipid uptake (Gregoire, 1991). Thus, the same factors that promote preadipocyte proliferation also promote preadipocyte development into adipocytes, ensuring that new preadipocytes replace differentiated preadipocytes (Figure 1).

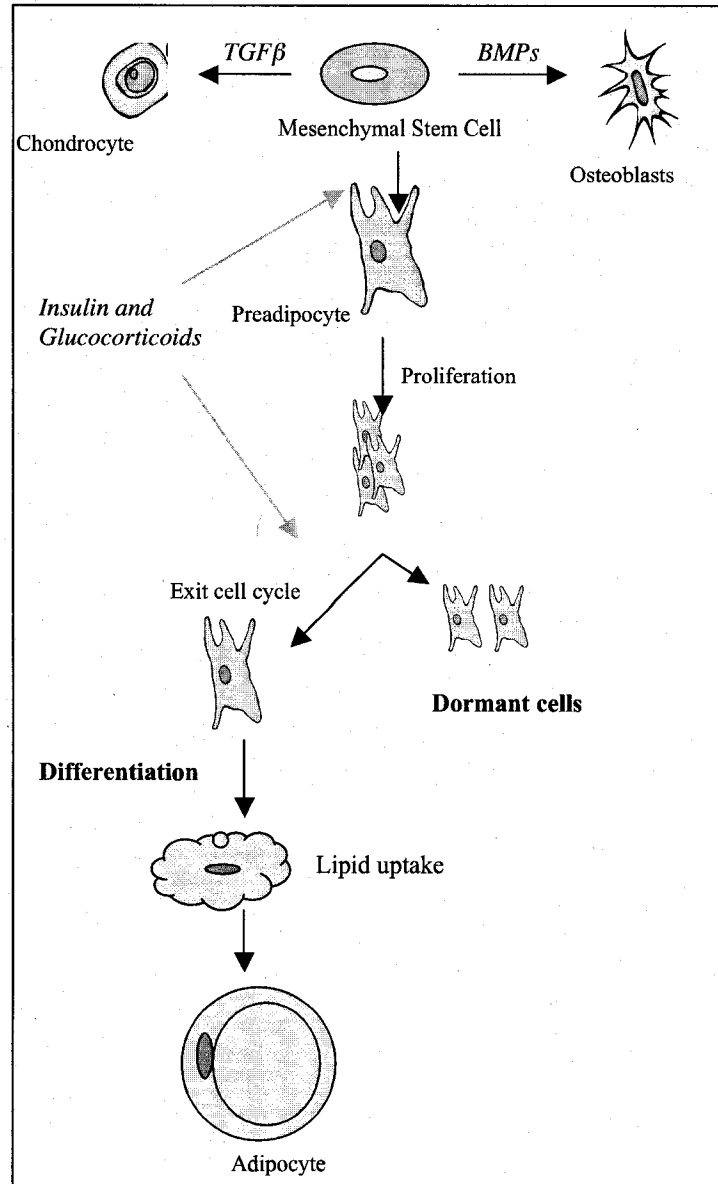


Figure 1. Preadipocyte differentiation begins after preadipocytes have proliferated. Mesenchymal stem cells can differentiate into chondrocytes, osteoblasts, and preadipocytes. Insulin and glucocorticoids initiates both preadipocyte proliferation and maturation.

Glucocorticoids may promote adipogenesis by down regulating the expression of Preadipocyte Factor-1 (Pref-1). Pref-1 is a transmembrane protein expressed in preadipocytes, but is not present in adipocytes (Smas, 1993). In order for adipogenesis to proceed, Pref-1 must be down-regulated (Boney et al., 1996; Lee, 2003; Smas, 1999). *In vitro*, exposure of preadipocytes to glucocorticoids decreases the expression of Pref-1, which is then followed by the activation of adipocyte transcription factors (see next section). Cultured preadipocytes expressing constitutively active Pref-1 do not undergo adipogenesis after exposure to glucocorticoids (Smas, 1999). Furthermore, mice expressing a soluble form of Pref-1 decrease adipocytes development as measured by the expression of adipocyte markers such as Fatty Acid Binding Protein (FABP4) and Leptin (Lee, 2003). However, the mechanism(s) that connect glucocorticoid signaling to Pref-1 expression is currently unknown.

The Activation of Transcriptional Regulators Promotes Adipogenesis.

Adipogenesis requires a complex activation scheme of adipocyte-specific transcriptional regulators (Table 1). First, the down-regulation of Pref-1 up-regulates the expression of transcription factors CCAAT enhancer binding protein (C/EBP) β and δ (Wu, 1996). Transcription factors C/EBP β and C/EBP δ up-regulate two other transcription factors: C/EBP α and peroxisomal-proliferator-activated-receptor γ (PPAR γ), both of which are required for adipocyte maturation (Zuo, 2006). Translation of C/EBP β and C/EBP δ promotes the transcription of C/EBP α and PPAR γ by binding to the promoters of these genes. Results of *in vitro* experiments demonstrate that blocking transcription of C/EBP β and C/EBP δ

results in a decreased expression of both C/EBP α and PPAR γ (Yeh, 1995). Northern blot analysis of PPAR γ and C/EBP α mRNA found that cells co-expressing C/EBP β and C/EBP δ displayed increased expression of PPAR γ compared to cells expressing C/EBP β or C/EBP δ , alone (Wu, 1996). Thus, these data suggests that C/EBP β and C/EBP δ act synergistically to activate the major transcriptional regulators of adipogenesis, C/EBP α and PPAR γ whose transcription is promoted after preadipocytes have exited the cell cycle (Salma, 2006).

C/EBP α plays a vital role in adipogenesis. Activation of C/EBP α promotes the expression of adipocyte genes such as FABP4, and GLUT4 (Lin and Lane, 1994). In culture, blocking the expression of C/EBP α by antisense C/EBP α RNA inhibits adipogenesis in cultured cells (Lin, 1992). Conversely, preadipocytes transfected with inducible C/EBP α have increased lipid accumulation and adipocyte-specific gene expression when C/EBP α is activated (Lin and Lane, 1994). These studies demonstrate that C/EBP α is a required regulator of adipocyte-specific gene transcription.

One major function of C/EBP α in adipogenesis is to promote the expression of PPAR γ in differentiating preadipocytes and mature adipocytes. PPAR γ is a nuclear hormone receptor consisting of two isoforms, PPAR γ 1 and PPAR γ 2 (Tontonoz, 1994). PPAR γ 2 is primarily expressed in adipocytes whereas PPAR γ 1 is found in adipocytes, hematopoietic cells, and endothelial cells. Both isoforms stimulate differentiation; however, PPAR γ 2 has greater effect on the differentiation of preadipocytes (Mueller, 2002; Tontonoz, 1994). In response to

ligand binding, PPAR γ forms an activated a heterodimer complex with the retinoid x receptor α (RXR α) (Tontonoz, 1994). The PPAR γ 2 and RXR α heterodimers recognize the ARF-16 binding site of adipocyte-specific genes (Mueller, 2002). These genes promote the metabolism of fatty acids in developing adipocytes (Morrison and Farmer, 2000). *In vitro*, fibroblasts transfected with PPAR γ 2 constructs accumulate lipid after exposure to conditions promoting differentiation (Tontonoz, 1994). Furthermore, the requirement for PPAR γ is illustrated by studies demonstrating that preadipocytes lacking PPAR γ do not differentiate (Zuo, 2006). For these reasons, PPAR γ is often referred to as the “master regulator” of adipocyte gene transcription due to its important role in adipocyte development.

The initiation of C/EBP α transcription promotes the transcription of PPAR γ (Elberg, 2000; Freytag, 1992; Tontonoz, 1994). Together these two transcription factors regulate the expression of one another by forming a positive feedback loop, promoting the transcription of genes expressed by adipocytes such as FABP4, LPL, GPDH, and GLUT4 (Hemati, 1997; Wu, 1999; Wu, 1995). The importance of C/EBP α and PPAR γ in adipogenesis can be seen when fibroblasts transfected with the two transcription factors take on an adipocyte phenotype in the absence of external differentiation signals (Tontonoz, 1994). However, fibroblasts transfected with C/EBP α and PPAR γ individually did not produce cells with an adipocyte phenotype. Thus, these experiments support the finding that C/EBP α and PPAR γ work together to increase the development of adipocytes and maintain the adipocyte phenotype.

Protein	Name	Role in Adipogenesis
Pref-1	Preadipocyte factor 1	Inhibits adipocyte gene transcription (Smas, 1999)
C/EBP α	CCAAT/ enhancer binding protein α	Promotes the transcription of adipocyte genes (Lin and Lane, 1994)
C/EBP β	CCAAT/ enhancer binding protein β	Promotes the transcription of C/EBP α (MacDougald, 1994)
C/EBP δ	CCAAT/ enhancer binding protein δ	Promotes the transcription of C/EBP α (MacDougald , 1994)
PPAR γ	Peroxisome Proliferator activated receptor γ	Promotes transcription of adipocyte genes (Tontonoz, 1994)

Table 1. Transcriptional regulatory proteins involved in regulating adipogenesis.

Wnt Signaling Helps Maintain an Undifferentiated Preadipocyte.

The dormancy of resident preadipocytes in adipose tissue is maintained by a variety of factors. One factor includes the Wnt signaling cascade which plays a role in cell growth and cell-fate determination in a variety of cell lineages and tissues (as reviewed in Honeycutt, 2004). The activation of the Wnt pathway inhibits adipogenesis (Longo, 2002; Ross, 2000) as measured by a decrease in lipid accumulation and expression of adipocyte markers such as FABP4 (Ross, 2000). Wnt signaling also inhibits adipogenesis by blocking the expression of major adipogenic transcription factors C/EBP α and PPAR γ (Gustafson and Smith,

2006). Therefore, the balance between Wnt and PPAR γ signaling determines whether or not adipogenesis will proceed (Liu and Farmer, 2004; Liu, 2006).

Expression of factors in adipose tissue and surrounding tissue can also inhibit adipogenesis. For example, the expression of tumor necrosis factor α (TNF α) (Qian, 2001), and interleukin-1 (IL-1) (Suzawa, 2003) inhibit adipogenesis. Decreasing TNF α and IL-1 expression in MSCs resulted in the down-regulation of Wnt, thus promoting adipogenesis in 3T3-L1 preadipocytes (Gustafson, 2006). *In vitro*, TNF α and IL-1 promote osteogenesis, an alternate differentiation pathway of MSC, through activation of NF- κ B and suppression of PPAR γ (Suzawa, 2003). A possible method to help prevent obesity would be to maintain preadipocytes in an undifferentiated state. In an undifferentiated state preadipocytes would not be able to accumulate lipid, and people would be less likely to become obese.

Adipose Tissue Growth Requires Angiogenesis.

To support adipose tissue growth, new blood vessels need to be formed in the tissue. The recruitment of endothelial cells to form vessels is called angiogenesis. Endothelial cell recruitment is initiated by angiogenic factors secreted by developing adipocytes. Endothelial cells proliferate and migrate to form new vessels off existing nerve-associated blood vessels (Neels, 2004). Evidence that angiogenic factors are secreted by developing adipocytes can be seen *in vivo* when preadipocytes injected subcutaneously into mice develop into vascularized adipose tissue at the site of injection (Neels, 2004). *In vitro*, the expression of angiogenic factors increases in preadipocyte cultures exposed to

conditions promoting differentiation (Hu, 1996). Thus, as adipose tissue expands, more angiogenic factors are released to stimulate new vessel growth.

Vascular endothelial growth factor (VEGF) is one such angiogenic factor secreted by adipocytes. VEGF promotes the development of new vessels by attracting endothelial cells to the area and promoting their proliferation (as reviewed in Neufeld, 1999). When expression of angiogenic factors such as VEGF are blocked *in vivo*, revascularization of the tissue is inhibited (Yamaguchi, 2005). *In vitro*, co-cultures of endothelial cells and preadipocytes promoted adipogenesis (Aoki, 2003). In preadipocyte culture, increasing VEGF concentrations in cell media increases the lipid uptake and expression of adipocyte-specific markers (Claffey, 1992). These investigative studies have shown that angiogenic factors directly promote adipogenesis. Thus, communication between angiogenic factors and preadipocytes are involved in adipocyte development.

Inhibiting the expression of angiogenic factors like VEGF suppresses adipogenesis. Inhibition of expression of angiogenic factors results does not support normal vessel growth and developing tissue cannot get enough nutrients to new tissue growth (Fukumura, 2003). Evaluation of VEGF mRNA expression in differentiating preadipocytes demonstrated that VEGF expression is increased during differentiation (Claffey, 1992). In cultures of preadipocytes and endothelial cells, addition of a neutralizing anti-VEGF antibody to culture inhibited both angiogenesis and adipogenesis (Nishimura, 2007). These studies indicate that communication between endothelial cells and adipocytes is

important for the development of blood vessels, which in turn is required to support new adipose tissue growth.

The Notch Signaling Pathway is One Mechanism in which Cells Communicate.

Communication among cells in adipose tissue is necessary for adipogenesis. The Notch signaling pathway is one mechanism which may facilitate cell to cell communication (Artavanis-Tsakonas, 1995). Notch is a cell surface receptor which is activated by one of its ligands to relay signals from the membrane of the cell to its nucleus. To date, components of the Notch signaling pathway have been found in all cell types (as reviewed in Artavanis-Tsakonas, 1999) and is required for such processes as neurogenesis (Nye et al., 1994), myogenesis (Shawber, 1996), angiogenesis (Zimrin, 1996), hematopoiesis (Bigas, 1998), and adipogenesis (Liu, 2003). Depending on the cell type and its environment, activation of Notch receptors may promote cell proliferation, differentiation, or apoptosis (Artavanis-Tsakonas, 1999; Morrison and Farmer, 2000; Zimrin, 1996).

Notch is a cysteine-rich single pass transmembrane protein receptor that is activated when the epidermal growth factor-like (EGF) repeats in the extracellular domain of the receptor interact with EGF-like repeats in the Notch ligand (Figure 2). Notch receptor and ligand interactions promote the first cleavage in the extracellular region of Notch, caused by a member of the ADAM protease family known as TACE (TNF α -converting enzyme) (Brou, 2000; Logeat, 1998; Mumm, 2000). The first cleavage in Notch then promotes a second cleavage in the transmembrane domain mediated by γ -secretase-like activity (Fortini, 2002). A

third proteolytic cleavage occurs in the intracellular region also caused by γ -secretase-like activity, resulting in the release of a section of the Notch intracellular domain (NICD) (Schroeter, 1998). Liberated NICD translocates to the nucleus where it interacts with a family of transcription factors belonging to the CSL family (CBF-1 in mammals, Suppressor of hairless in *Drosophilla*, and Lag2 in worms) (Jarriault, 1998; Gupta-Rossi, 2004). CSL proteins are transcriptional repressors that become activators of transcription when they interact with NICD (Kopan, 1996). CSL-NICD interactions result in the transcription of a family of basic helix-loop-helix DNA binding proteins that include Hairy enhancer of split (HES) and Hairy enhancer of Split Related Protein (HERP) (Jarriault, 1995; Jarriault, 1998; Schroeter, 1998; Shawber, 1996). Thus, the activation of Notch signaling promotes the transcription of gene regulatory proteins that ultimately change the fate of the cell.

In mammals, the Notch family consists of four homologous receptor proteins. The Notch signaling cascade becomes activated after binding to one of its ligands, which includes two classes of transmembrane proteins: Delta and Serrate/ Jagged (Jarriault, 1998; Gupta-Rossi, 2004). Ligand type can determine which way a Notch-induced pathway is regulated. For example, NIH 3T3 cells expressing soluble Jagged-1 impairs cell migration due to alteration in FGF signaling, whereas NIH 3T3 cells expressing soluble Delta-1 do not impair endothelial cell migration (Zimrin, 1996; Panin, 1997; Trifonova, 2004). Despite these observations, the mechanisms how each ligand exerts different effects on the same pathway remain unclear.

Some studies suggest that ligand and receptor interactions cause a cleavage in the intracellular domain of the ligand, which translocates to the nucleus (Ascano, 2003; Bland, 2003; Ikeuchi and Sisodia, 2003). Evidence that a cleavage occurs in the intracellular Notch ligand domain is seen in neuroblastoma N2a cells expressing mouse Delta-1 and in NIH 3T3 cells expressing human Jagged-2. These cells contained a presenilin-dependent γ -secretase which causes proteolysis of the Notch ligand. In these studies, the cleaved portion of the ligand was found in the nucleus of these cells following proteolysis (Ikeuchi, 2003; Kolev, 2005). The possible genes affected by the translocated ligands could have control over cell fate, however no downstream effectors have been identified to date.

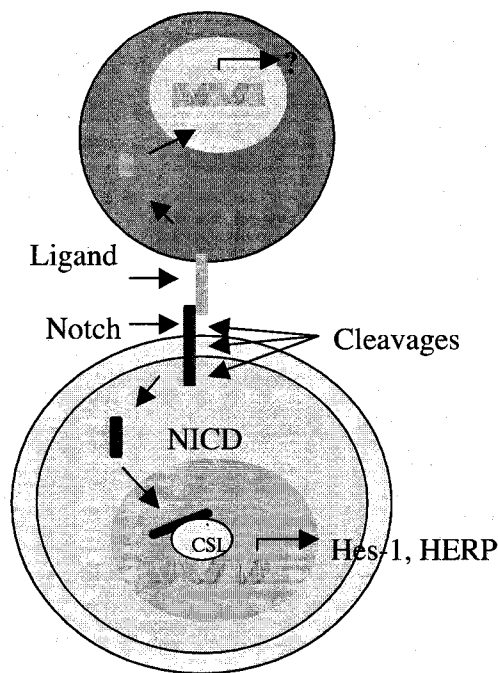


Figure 2. Notch signaling requires interactions between membrane bound ligands. Canonical Notch signaling requires that the receptor expressed on the surface of one cell interacts with ligands expressed on the surface of an adjacent cell. These cleavages result in proteolytic cleavages which occur in Notch at the positions indicated. Cleaved Notch intracellular domain (NICD) travels to the nucleus of the cell and binds with CSL factors to help promote transcription of genes such as Hes-1 and HERP which regulates other gene targets. Similar cleavages have recently been observed in the intracellular domain of Notch ligands, however it is not known if the cleavage product promotes gene transcription.

The Ligand Jagged-1 has a Role in a Number of Cell Processes.

Jagged-1 is one of the mammalian ligands for Notch receptors (Lindsell, 1995). To date, there have been two Jagged ligands identified. Jagged-1 has been detected in a variety of mammalian tissues including lymph nodes, bone marrow, skin epithelia, olfactory epithelia, tooth buds, dorsal root ganglia, and the thymus

(Shawber, 1996). Jagged-1 and Notch interactions are involved in many signaling pathways including angiogenesis. For example, Jagged-1 and Notch interactions promote FGF recruitment of endothelial cells (Zimrin, 1996). In murine embryogenesis, homozygous mutations in the Jagged gene cause lethal deformities in vessel formation in the embryo and placenta, resulting in massive hemorrhage (Xue, 1999). Heterozygous Jagged-1 mutations in mice also cause abnormalities in vessel development (Kamath, 2004). These data support a requirement for Jagged-1 in the development and maturation of the vasculature.

Mutations in Jagged-1 can also disrupt the development and function of a number of cell types. Alagille syndrome, which is the result of a gene mutation in Jagged-1 causing non-functional forms of the ligand, is characterized by deformities in epithelial cells affecting the structure and function of the liver, eyes, heart, and vertebra (Kamath, 2004). Many people with the Alagille syndrome have deformities in the cardiovascular system as well. These deformities can result in aneurysms, increasing the incidence of morbidity (Kamath, 2004). Children with Alagille syndrome have a reduced percentage of body fat and are underweight due to abnormal adipocyte development and poor absorption of fat (Wasserman, 1999). While other factors are probably involved in the disease's progression, the syndrome provides strong evidence of the importance of Jagged-1 expression in many tissues, including adipose.

Notch and Jagged-1 Interactions are Necessary for Adipogenesis.

Notch signaling regulates the differentiation of preadipocytes into adipocytes (Garces, 1997; Ross, 2004; Ross, 2006). Researchers have observed

less lipid accumulation in 3T3-L1 preadipocytes expressing antisense Notch-1, compared to control cells (Garces, 1997). In these cells, RT-PCR confirmed that expression of PPARs decrease as the concentration of recombinant antisense Notch-1 protein increases in 3T3-L1 cell cultures (Garces, 1997). In murine ST-2 stromal cells and in MC3T3 cells with increased expression of Notch intracellular domain differentiated into adipocytes over the expected osteoblasts lineage even after exposure to varying levels of osteogenesis promoting factors including cortisol (Sciaudone, 2003). These results suggest that Notch signaling is affects preadipocyte differentiation through the activation of transcription factors belonging to the PPAR family.

Conversely, Notch activation also inhibits adipogenesis *in vitro*. Activation of endogenous Notch receptor by exposing 3T3-L1 preadipocytes to large quantities of immobilized Jagged-1 protein results in a decrease lipid uptake and adipocyte-specific gene expression (Ross, 2004). Notch ligands also promote the differentiation of stem cells into adipocytes over osteoblasts (Nobta, 2005). Thus, constitutive Notch signaling may also inhibit adipocyte development.

These seemingly conflicting observations indicate that a balance of Notch activation might be needed for adipogenesis to occur normally. Notch activation promotes and inhibits preadipocyte differentiation (Garces, 1997; Ross, 2004). Investigations have focused on uncovering possible mechanisms that regulate adipogenesis via activated Notch. One such suspect is the DNA binding protein Hes-1. Hes-1 is a known target gene of Notch signaling, activated when the NICD interacts with CSL proteins in the nucleus (Figure 3) (Jarriault, 1995).

Hes-1 has been determined to have dual roles in adipogenesis. In 3T3-L1 preadipocytes with knocked-down Hes-1 expression have decreased lipid accumulation and FABP4 expression. 3T3-L1 preadipocytes constitutively expressing Hes-1 have less lipid accumulation and FABP4 expression. Cells with constitutive Hes-1 expression inhibited expression of C/EBP α , and PPAR γ . This suggests that Hes-1 inhibits adipogenesis and that Hes-1, activated by Notch signaling, regulates adipogenesis at a point upstream of C/EBP α and PPAR γ expression.

Another point of regulation upstream of C/EBP α and PPAR γ is Pref-1, which as previously discussed, regulates preadipocyte differentiation (Wu, 1996). Hes-1 is upstream of Pref-1 and may regulate adipogenesis through inhibiting the down regulation of Pref-1 expression (Ross, 2006). These experiments suggest that Notch activation is regulating adipogenesis through Hes-1.

In conclusion, a role for Notch-1 in adipogenesis has been reported. The specific roles of the Notch ligands in adipogenesis are unknown, although studies have also determined that Notch activation, via interactions with Jagged-1, can affect adipogenesis. Furthermore, Jagged-1 is down-regulated in 3T3-L1 preadipocytes exposed to differentiation media (Small, unpublished). Since the identity of the activating ligand has been demonstrated to be important in the Notch signaling response in several cell lines and tissues (Trifonova, 2004), it is possible that unknown if Jagged-1 and other ligand expression can exert effects on preadipocyte differentiation. Therefore, we looked specifically at the effects of constitutive expression of Jagged-1 in 3T3-L1 preadipocytes after they were

induced to differentiate (Garces, 1997; Ross, 2004; Ross, 2006). We found that constitutive expression of Jagged-1 inhibited normal adipogenesis in NIH 3T3-L1 cells in culture and *in vivo*, supporting the conclusion that Jagged-1 is an important regulator of adipogenesis and adipose tissue development.

Hypothesis

Constitutive expression of Jagged-1 in preadipocytes will inhibit adipogenesis *in vitro* and adipose tissue development *in vivo*.

Specific Aims

1. Determine effects on adipogenesis in preadipocytes constitutively expressing Jagged-1 *in vitro*.
2. Determine the ability of preadipocytes constitutively expressing Jagged-1 to form fat pads *in vivo*.

Objectives

To meet these aims, we examined the effects that constitutive expression of Jagged-1 had on the L1 phenotype in a cell culture system. In order to determine if inappropriate Jagged-1 expression altered characteristics that are associated with adipocyte differentiation, we examined the growth/ survival kinetics, lipid up-take, and expression of PPAR γ and FABP4 in experimental and control cells. To determine if these cells are deficient in adipogenesis *in vivo*, we compared fat pads that developed from injection of experimental and control 3T3-

L1 preadipocytes. Fat pads were evaluated for average adipocyte size, adipocytes number, and vessel formation. We found that increased expression of Jagged-1 in 3T3-L1 preadipocytes impaired preadipocyte differentiation as determined by changes in lipid uptake, expression of adipocyte-specific genes *in vitro*, and *in vivo* the formation of fat pads in nude mice.

CHAPTER II

MATERIALS AND METHODS

In Vitro Protocol

L1 Control/ L1J1 Cell Lines

NIH 3T3-L1 preadipocytes were chosen for this study because they are immortalized cells that can be easily propagated and differentiate into adipocytes when exposed to conditions promoting adipogenesis (Green, 1976). Briefly, NIH 3T3-L1 cells were stably transfected to constitutively express Jagged-1 (L1J1). A pcDNA3.1+G418 cDNA vector (Invitrogen) containing the cDNA gene encoding full length human Jagged-1 was stably transfected into 3T3-L1 cells using Fugene 6 (Small, 2001). The vector also contained a Genticin resistant gene so that cells which incorporated the vector could be isolated after addition of this antibiotic to the growth media. The control cell line was developed by transfecting the empty vector into NIH 3T3-L1 cells (L1). Stable transfectants were obtained from individually isolated cells from clonal populations of Genticin (G418) resistant cells. To determine that cell line clones expressed exogenous Jagged-1 constructs, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed using human Jagged-1 specific primers (Table 3.).

Cell Culture

L1 and L1J1 cells were plated in 6-well plates with 3mls of L1 media [Dulbecco's Modified Eagle's Medium (DMEM) with 10%(v/v) fetal bovine

serum (FBS), and 1% (v/v) antibiotic and antimycotic], or L1 media containing Gentecin (DMEM with 10% (v/v) FBS, 1% (v/v) antibiotic and antimycotic, and 0.8mg/ μ g Gentecin) respectively, until samples reached confluence. Once confluent, cells were exposed to differentiation media [DMEM with 10% (v/v) FBS, 1% (v/v) antibiotic and antimycotic, 1 μ g/ml Dexamethazone, 10 μ g/ml IBMX (3-isobutyl-1-methylxanthine), 10 μ g/ml insulin] for 48 hours. After 48 hours, differentiation media was replaced with maintenance media [DMEM with 10% (v/v) FBS, 1% (v/v) antibiotic and antimycotic, 10 μ g/ml insulin] for the remainder of the experimental period.

GFP Cell Lines

In order to track L1 control cells and L1J1 cells for our *in vivo* studies, a green fluorescent protein (GFP) was added to cell lines to be able to distinguish cells that successfully take up the vector from cells that do not. Briefly, a DNA molecule encoding a hygromycin and chloramphenicol resistant gene (pExchange Module EC-Hyg) was cloned into a GFP vector (pIRES-hrGFP-1a) using Cre Recombinase. To make stable cell lines expressing GFP protein, L1 and L1J1 cells were transfected with the construct pIRES-hrGFP-Hygro. These cells were then grown in cell culture (see Appendix A.).

Clonal populations were developed from isolated L1 and L1J1 cells containing GFP. To determine that clones contained the desired construct, clones grown on glass cover slips were prepared using the Anti-fade Prolong[®] protocol. These slides were observed under a fluorescent microscope. Cell that contained

the GFP vector were fluorescent. Clones with notable fluorescence (Figure 3) were selected for the remainder of the experiment.

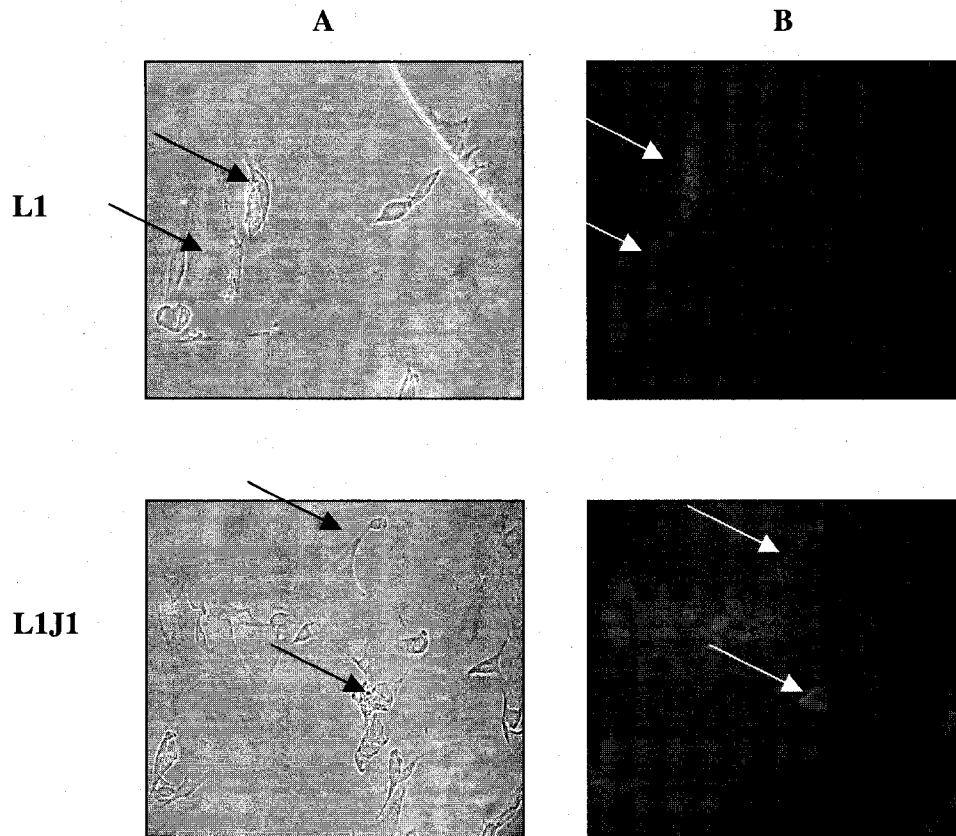


Figure 3. GFP positive cell lines were developed from individual cells expressing GFP. L1 and L1J1 cells containing GFP pIRES-GFP-Hygro were grown on coverslips. Images taken at 200X magnification were used to identify colonies expressing GFP (column A). Slides were observed for GFP expression under fluorescence 520nm (column B). Arrows point to a few selected cells which had GFP expression.

Population Curves

L1 control and L1J1 cells were grown in culture in L1 media (as previously described), or in conditions promoting differentiation (as previously

described). Initially, 10,000 cells were plated in 12 well plates. Cells were harvested 24 hours, 48 hours, four days, seven days, ten days, and two weeks after initial plating. During this period, media was changed every 48 hours. For harvest, cells were rinsed with PBS after removal of media and exposed to 1ml trypsin to detach cells from culture-ware. Collected cells were re-suspended in a dilution of cell suspension media and trypan blue, and counted on a hemocytometer to determine the number of cells per well. Population curves were also determined after L1 control and L1J1 cells were exposed to conditions promoting differentiation. For these cells, 10,000 cells per well were plated in a 12 well plate and left to grow until confluent. Once confluent, 1ml of differentiation media was added to each well. Cells were then collected and counted as previous mentioned. Three replications for each sample were taken to determine the average population growth rate.

Oil-red-O Staining

Oil-red-O binds to long chain hydrocarbon tails of triglycerides and is used to evaluate lipid accumulation in differentiating and mature adipocytes, as described (Sorisky, 1996). For Oil-red-O staining, media was aspirated from cell cultures and cells were washed twice with 1X PBS. Cells were fixed in culture dishes by exposing them to 2ml of neutral formalin for ten minutes. Formalin was then discarded and plates were rinsed twice with 1X PBS to remove excess fixative. 2ml Oil-Red-O solution (60ml/100ml v/v of a 0.5% stock solution of Oil-red-O) was added to each culture and left to gently agitate for at least 30 minutes. After 30 minutes, Oil-Red-O was removed and cultures were rinsed

with 1X PBS. Staining was preserved by adding 2ml of a glycerol solution (60% glycerol and 40% water) to each culture dish.

Oil-red-O staining was also used to qualitatively compare lipid accumulation after varying the exposure to differentiation conditions. L1 control and L1J1 cell cultures were exposed to conditions promoting differentiation for 48 hours, four days, ten days and two weeks. After these time periods, cultures were stained with Oil-red-O as previously described. All cultures were compared to control cultures that were not exposed to differentiation conditions. Phase contrast micrographs were taken at 200X magnification using an Olympus microscope.

L1 and L1J1 Cells Collection Points	Oil-Red-O Staining	RNA Isolation	Protein Isolation
Non Differentiated	X	X	X
24 Hour Exposure to Conditions Promoting Differentiation	X	X	X
48 Hour Exposure to Conditions Promoting Differentiation	X	X	X
7 Day Exposure to Conditions Promoting Differentiation	X	X	X

Table 2. *In vitro* procedures used to characterize L1 control and L1J1 cell populations.

RNA Isolation

Cells were collected mechanically by scraping after being grown in culture for desired periods of time. Cells were pelleted by centrifugation at 1,500rpm for five minutes at 4°C. After media was removed, the cell pellets were re-suspended in 5ml of 1X PBS to remove excess media and serum. Pellets were washed 2X and centrifuged again. PBS was aspirated and the pellets were then stored at -80°C, or immediately used for total RNA isolation.

The TriReagent protocol (Sigma) was used to isolate total RNA from cell pellets (see Appendix B.). Optical densities of the samples were measured at a wavelength of 260nm to determine mRNA concentrations. To check for quality of isolated RNA, samples were separated for visualization using a formaldehyde gel. Messenger RNA (mRNA) was isolated from total RNA using the Oligotex mRNA Spin-Column Protocol (Qiagen) so that cDNA could be made (see Appendix C.).

RT-PCR cDNA was produced from the mRNA samples by reverse transcriptase synthesis (Promega). For each mRNA sample, 0.5µg of primer (oligo dT) was added to 4µl of mRNA template. Samples were briefly centrifuged and incubated in a thermocycler (PTC-100™) for five minutes at 70°C, then 4°C for 5 minutes. A master mix of Improm-II™ reaction buffer (1X of a 5X solution), MgCl₂ (3mM), dNTP (0.5mM), RNase inhibitor (1u/µl), Improm-II™ reverse transcriptase (1µl), and sterile water was prepared for each sample. Primer and mRNA mix was added to 15µl of master mix. Samples were briefly centrifuged and incubated in a thermocycler for fifteen minutes at 25°C,

followed by 60 minutes at 40°C. 2µl of RNase was then added to each sample and incubated at 37°C for 30 minutes to remove template mRNA. Following this procedure, 80µl TE buffer was added to a final volume of 100µl.

Amplification was performed on cDNA using primers specifically designed (Integrated DNA Technology) for the genes of interest. 5µl of cDNA was added to 45µl of master mix [2mM MgCl₂ (25mM), 0.2mM dNTP (10mM), and 1µL Promega® Taq polymerase], which also contained 10µM of the desired upstream primer and 10µM of the desired downstream primer. Samples were denatured, (94°C for 30 seconds and one minute on the last cycle), annealed (30 seconds at 55°C) for 30 cycles, and followed by polymerization (1 minute at 72°C) after the last cycle. cDNA samples were stored at 4°C. PCR products were run through a gel to visualize separation and determine the presence of the gene of interest. Electrophoresis was performed using a 1% agarose gel and 1X Tris acetate EDTA buffer. Each sample was loaded into the gel after having been mixed with 10µl of DNA dye (xylene cyanol and bromophenol suspended in a 50:1 ratio of formide to 5M EDTA pH 8). Gels were then stained with ethidium bromide and observed under an ultraviolet light. Images of gels were captured on an ultraviolet light box.

Primer	Sequence
Human Jagged-1 Sense	5'-CGG CCC ACA TGC TCT ACA-3'
Human Jagged-1 Anti-sense	5'-CGG GAA GAC AGT CGC AGT-3'
FABP4 Sense	5'-TGA TGC CTT TGT GGG AAV CTG -3'
FABP4 Anti-sense	5'- CTT TAA AAA AAA CAA TAA ATC CGA CTG ACT -3'
PPAR γ Sense	5'-TTG AGT GCC GAG TCT GTG GGG ATA A-3'
PPAR γ Anti-sense	5'-AGG GAG GCC AGC GTG TAG A-3'
LPL Sense	5'-GTC ACC TGG TCG AAG TAT TG-3'
LPL Anti-sense	5'-TTG TAG GGC ATC TGA GAG CGA GTC-3'
B-Actin Sense	5'- TAA GGC CAA CCG TGA AAA GAT GAC -3'
B-Actin Anti-Sense	5'- ACC GCT CGT TGC CAA TAG TGA TG -3'

Table 3. Primer sequences used in this study.

Protein Isolation

Separate extractions of cytoplasmic and nuclear protein were performed on proteins extracted from the cells. Cells were mechanically collected from culture. Using the Nuclear Extraction Kit (Imgenex), cell cultures (grown in conditions as previously described) were washed with 5ml of cold PBS/PMSF buffer (10X PBS, 100mM PMSF, ddH₂O), aspirated, and washed again. Cells were collected mechanically by scraping and added to a 15ml conical tube and centrifuged for five minutes at 1,000rpm at 4°C to isolate proteins from other cell structures. The resulting pellet was kept on ice after the supernate was discarded. Next, 1ml of 1X Hypotonic buffer was used to re-suspend the fraction. To isolate the cytoplasmic proteins from other proteins in the fraction, samples were transferred to a chilled microcentrifuge tube and incubated on ice for 15 minutes. 50 μ l of a detergent solution was added to each sample, and samples were

vortexed. Samples were centrifuged for 30 seconds at 14,000rpm at 4°C, and the resulting supernatant, which represented the cytoplasmic fraction, was transferred to a chilled microcentrifuged tube and stored at -80°C. The pellet which represented the nuclear fraction was re-suspended in 100µl Nuclear Lysis Buffer (10mM DTT, Nuclear Ext. Buffer, and 100X PIC) and vortexed. Samples were incubated and rocked for 30 minutes at 4°C. Samples were vortexed and centrifuged for ten minutes at 14,000rpm at 4°C. The nuclear cell lysate contained the supernate which was transferred to a chilled microcentrifuged tube and stored at -80°C.

To determine protein concentration of cytoplasmic protein, a bicinchoninic acid assay (BCA assayTM Pierce, Rockland, Illinois) was performed on all cytoplasmic protein samples. Each sample was added to a working reagent at a ratio of 1:20 (working reagent was a 1:50 dilution of reagent A, consisting of 500ml sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1M sodium hydroxide; reagent B contained 4% cupric sulfate) was combined in a microplate. Plates were incubated for 30 minutes at 37°C and optical densities were determined at a wavelength 564nm on a plate reader. Protein samples were compared to known BSA concentrations (measured at 1000, 500, 250, and 125µg/µl). Nuclear protein concentrations were determined using DcI assayTM (Pierce). 5µl of each sample was added to a well in a 96 well microplate. 20µl of reagent S (SDS), 25µl of reagent A (sodium hydroxide), and 200µl of reagent B (Folin Reagent) was added to each well. Plates were incubated for 15 minutes at room temperature and optical densities were

determined at a wavelength 750nm on a plate reader. All samples were read in triplicate for BCA and D_CI assays.

Proteins were separated by sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) using the method of Laemmli (Laemmli, 1970). Nuclear protein and cytoplasmic protein concentrations were normalized to one another to determine the volume of protein that should be used for each gel. Final protein volumes were determined by number of wells in each gel. Protein and running buffer (1:4) was prepared and boiled for five minutes. SDS-PAGE gels were prepared for electrophoresis with 15%, 12%, 10%, or 8% acrylamide depending on the size of the protein of interest. Gels were run using the SDS-PAGE method on a Dual Electrophoresis System (Owl, Scientific, Inc), at 32-90 V. 1X SDS-PAGE buffer (1.03g/L Tris, 14.4g/L glycine) was added to the electrophoresis unit before each run.

Separated proteins were transferred from the gel to supported nitrocellulose (Hybond-C extra Amersham Biosciences) for immunostaining. Blotting paper was soaked in Bjerrum and Schafer-Nielsen (1986) buffer (48mM Tris, 192mM glycine, 20% methanol) for five minutes. Gels and nitrocellulose were sandwiched between blotting paper and locked into transfer unit cassettes which were then loaded into the transfer electrophoresis unit TE 62 (Amersham Bioscience). Transfers were performed in Bjerrum and Schafer-Nielsen buffer at 1000amps for 60 minutes. The transfer unit was packed in ice to keep cool. Membranes were soaked in Poncaeu stain briefly following transfer to observe

protein transfer on the membrane. After visualization, Poncaeu stain was removed by rinsing.

Non-specific binding on membranes was blocked using 5% dry non-fat milk in 0.1% Tris-buffered saline (TBS-8g Sodium Chloride, 20ml 1M Tris HCL diluted to 1000ml with distilled water, pH 7.6), 0.1% Tween 20, and 0.1% sodium azide for at least one hour or overnight. Primary antibodies (dilution recommended by manufacturer) were added to the 5% milk with 0.1% azide, and rocked overnight at 4°C. Primary antibodies to PPAR γ (Gene Tex Inc.), and FABP4 (R&D Systems) were used as markers for differentiated adipocytes; whereas the antibody to Pref-1 was used as a marker for preadipocytes (Table 4).

Primary Antibody	Secondary Antibody	% Acrylamide Gel
FABP4 1: 500	Anti-Goat 1:20,000	12%
PPAR γ 1000	Anti-Rabbit 1:20,000	10%
Pref-1 1:500	Anti-Mouse 1:20,000	12%
Lamin 1:1,000	Anti-Mouse 1:20,000	10%

Table 4. Antibodies used for immunoblot analysis.

After incubation, membranes were washed four times with 0.1% TBS with 0.1% Tween 20 (1X TTBS) to remove unbound antibody. Next, blots were incubated in 5% milk with corresponding HRP (horseradish Peroxidase conjugate) secondary antibody (dilutions recommended by manufacturer, Rockland), for one hour at room temperature. The membranes were then washed four times with 1X TTBS to remove excess secondary antibody. Following the manufacturer's protocol, 4mls of ECL reagent (Amersham Bioscience) was added to each membrane and agitated for 30 seconds to five minutes. Excess moisture was removed from the membranes and wrapped in Saran Wrap®. The membranes were then exposed to film and developed manually. Films were exposed to the membrane for one minute, five minutes, or longer if necessary. Autoradiographs were scanned and the images were imported and saved for evaluation.

Lastly, membranes were stripped to re-probe for other proteins of interest. Membranes were not stripped more than three times. For stripping, the membranes were soaked in stripping buffer (100mM B-Mercaptoethanol, 2% SDS, 62.5mM Tris-HCL pH 6.7) at 60°C for 15 minutes and then agitated at room temperature for 15 minutes. The membranes were then washed three times with 1X TTBS and blocked again for non-specific protein binding.

In Vivo Protocols

Animal Injection

Stable Jagged-1 transfectants were grown in cell culture. Once confluent, cells were trypsinized and collected as previously described. Cell suspensions

were centrifuged and re-suspended in 1X PBS. An aliquot of cell suspension was placed on a hemocytometer to determine cell concentration. For the first experiment, 2.5 million cells were collected; for the second experiment four million cells were collected. Cell suspensions were mixed in a 1:1 ratio with Matrigel (R&D Systems) or Matrigel with reduced growth factors as indicated. Mixtures were collected into a syringe and kept on ice until injected.

Male athymic nude mice, between six to eight weeks old, were housed in environmentally controlled housing at Maine Medical Center Research Institute (MMCRI). Mice were injected with either a mixture of L1 control cells, or L1J1 cells with Matrigel to support new tissue growth (Kawaguchi, 1998). A 25 gauge needle was used to inject 250 μ L of the mixture subcutaneously into the right flank of each mouse. The first experiment, four mice were injected with either L1 control or L1J1 cell suspension (mixed with Matrigel and growth factors) and for the second experiment six mice were used for each cell type (Table 4). Mice were only handled under a sterile flow hood, and surfaces were cleaned with ethanol between exchanges.

Mice were observed daily for fat pad growth. Fat pads were left to grow for four weeks. After four weeks of growth, mice were terminated by spinal dislocation under anesthesia. Fat pads were removed along with epididymial fat and kidneys which were used as control tissues. A portion of each tissue was collected for immunohistochemistry (IHC), or protein and RNA isolation. Prior to the termination in the second experiment, 200 μ L of a warmed BrDu solution was injected into mice 16 hours and 1 hour prior to being euthanized. Injections

were given subcutaneously above the right leg. The purpose of these injections was to evaluate proliferating cells in sections of fat pads.

Experiment Number	Number of Mice Used	Number of Cells injected per mouse	Growth Factors Used	Time of Growth
1a	8	500,000	Regular	4 weeks
1b	8	500,000	Reduced	4 weeks
2	12	2,500,000	Reduced	4 weeks

Table 5. Parameters for *in vivo* experiments.

Histochemistry

Half of each fat pad was fixed for paraffin embedding. Tissues were placed in a cassette and fixed for 48 hours in 10% buffered formalin (dehydrated). Sections were prepared in the histology department care of MMCRI. Briefly, after dehydration, fat pads were infiltrated with Paraplast PlusTM embedding medium overnight (Oxford Ladware, St. Louis, MO) in the Tissue Tex Automatic Tissue Processor (Miles Inc., Diagnostic Division, Tarrytown, NY). Embedded fat pads were sectioned at 5 microns and were mounted onto glass slides.

Slides were stained with either hemotoxylin and eosin, or mason trichrome to observe basic tissue structure. Photomicrographs were taken of prepared slides at 200X magnification to observe cell number and size. To evaluate cell number in each fat pad, the number of cells within a given area of fat pad was recorded.

Within the same given area the size the fat cells was outlined and the average cell size per given area, was determined (three measurements per slide). Measurements were determined using Spot Imaging Software 4.6 (Diagnostic). Additional slides were exposed to antibodies against PECAM-1 a marker of endothelial cells (see Appendix D.). To determine relative PECAM-1 expression in the tissue photomicrographs were taken using Spot Imaging Software, and positive areas of PECAM-1 staining in these images were analyzed using Scion imaging. PECAM-1 was reported as areas with positive stain as an orange precipitate (determined after comparing positive stained slides to the negative PECAM-1 slides) to total fat pad in the field of vision.

Run Number	Histology Protocol
1	Hematoxylin and Eosin PECAM-1
2	Hematoxylin and Eosin Mason Trichrome PECAM-1 BrDu

Table 6. Histological procedure used to evaluate fat pad sections.

MRI

Magnetic Resonance Imaging (Zimrin, 1996) was conducted on the mice from the second experiment to assess development of tissue development. MRIs for each mouse at two weeks and three weeks after injections were taken at Maine Medical Center Research Institute Small Animal Imaging Core using a 7.0T

Bruker Pharmascan Magnet. Mice were sedated with 3% inhaled isoflurane and were imaged using the MRI to examine fat pad structure. Warm air was supplied to keep the mice warm during the MRI. MRI analysis was done using Paravision Image Display and Processing. A 40mm x 40mm field of vision was used to take images of the mice. Nine to thirteen 1mm section images were taken of sagittal, coronal, and axial views of the mice. A SAM PC Monitor (Small Animal Instruments Inc) was attached to the mice during the MRIs to observe respiration (breath/ minutes). Areas of interests were observed for fat content.

Statistics

To determine differences between fat pads, one way factorial analysis of variance (ANOVA) was performed on all quantitative data gathered. Data are expressed as the mean \pm standard error. Values of $P < 0.05$ were considered statistically significant.

CHAPTER III

RESULTS

Constitutive Expression of Jagged-1 in 3T3-L1 Preadipocytes causes a Delay in Adipogenesis, *In Vitro*.

Increased Expression of Jagged-1 Changes the Growth Dynamics of 3T3-L1 Preadipocytes.

Notch signaling regulates cell growth and survival (Artavanis-Tsakonas, 1995). Therefore, we examined population survival rates of adherent L1 control and L1J1 transfectants, as described under materials and methods, to determine if constitutive expression of Jagged-1 affects the growth dynamics of the L1 preadipocyte. The survival curves of L1 control cells differed from L1J1 cell lines, with the survival rate for L1 control cells 2.04 fold greater than that of L1J1 cells after 2 weeks of growth in culture (Figure 4A.). Total L1 control cell numbers were 18,000 cells/well compared to total L1J1 cell concentrations of 12,000/well cells after 24 hours of growth (Figure 4A). After four days of growth, total L1 control populations were 4.3 fold greater than total L1J1 cells, with 371,000 cells/ well compared to 87,000 cells/ well, respectively (Figure 4A). Total L1 control populations doubled soon after initial plating and entered log phase 48 hours after plating, whereas total L1J1 populations lagged behind L1 control cells, and did not reach log phase until seven days after plating in growth media. Both cultures exhibited a

spike in growth, followed by a gradual decrease in population numbers; however L1J1's peak population occurred about three days after L1 control cultures.

Upon exposure to differentiation media, which contains insulin and glucocorticoids, a similar trend was seen in the growth dynamics of L1 control versus L1J1 cells. The L1 control population's doubling time occurred at a higher rate than L1J1 cell lines. At four days exposure, L1 control cell survival rates totaled 8,139,000 cells/ well, which was 2.7 fold greater than L1J1 populations (Figure 4B). L1J1 cells reached a gradual population peak by two weeks with a total of 2,943,000 cells/ well (Figure 4B). After two weeks, L1 control populations began to decrease, while the L1J1 cell survival rate climaxed. The marked decrease in adherent L1 control cells was likely due to either cell death in L1 control cultures or the loss of mature cells that were no longer adherent to culture-ware.

In summary, the population curves of differentiating cells, similar to the population curves of cells in growth media, indicate that L1J1 cells exhibit slower growth and survival dynamics compared to L1 control cells. Under both concentrations, L1 control population numbers were greater at each collection point than L1J1 populations, with the only exception of L1J1 cell numbers at the 2 week collection point in L1J1 cultures exposed to differentiation media.

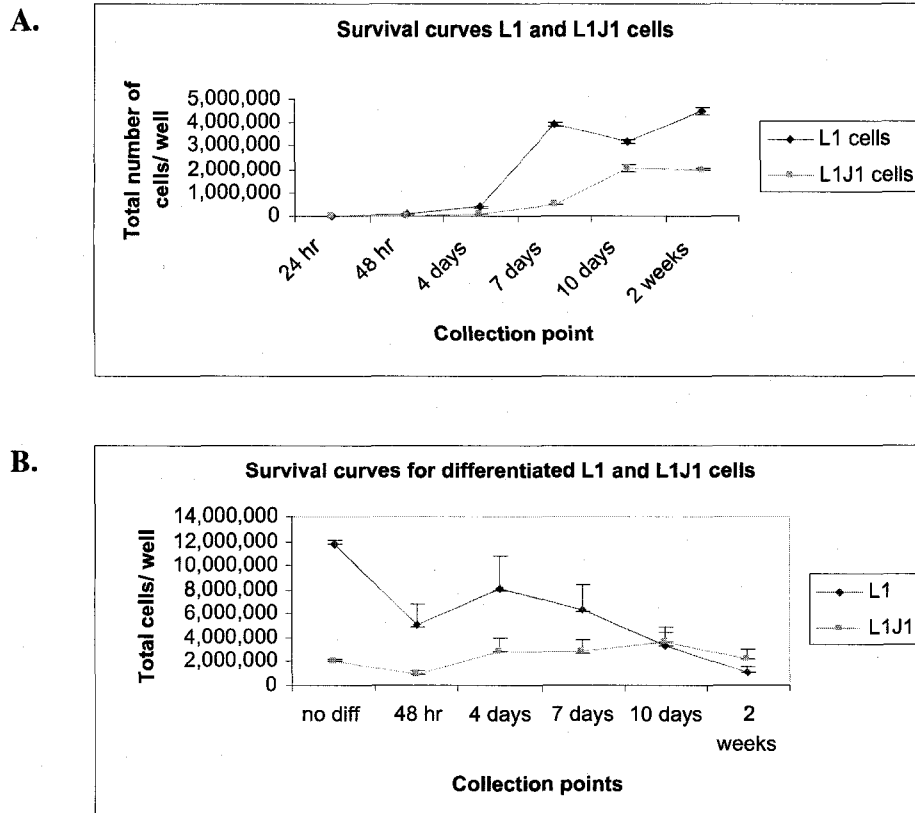
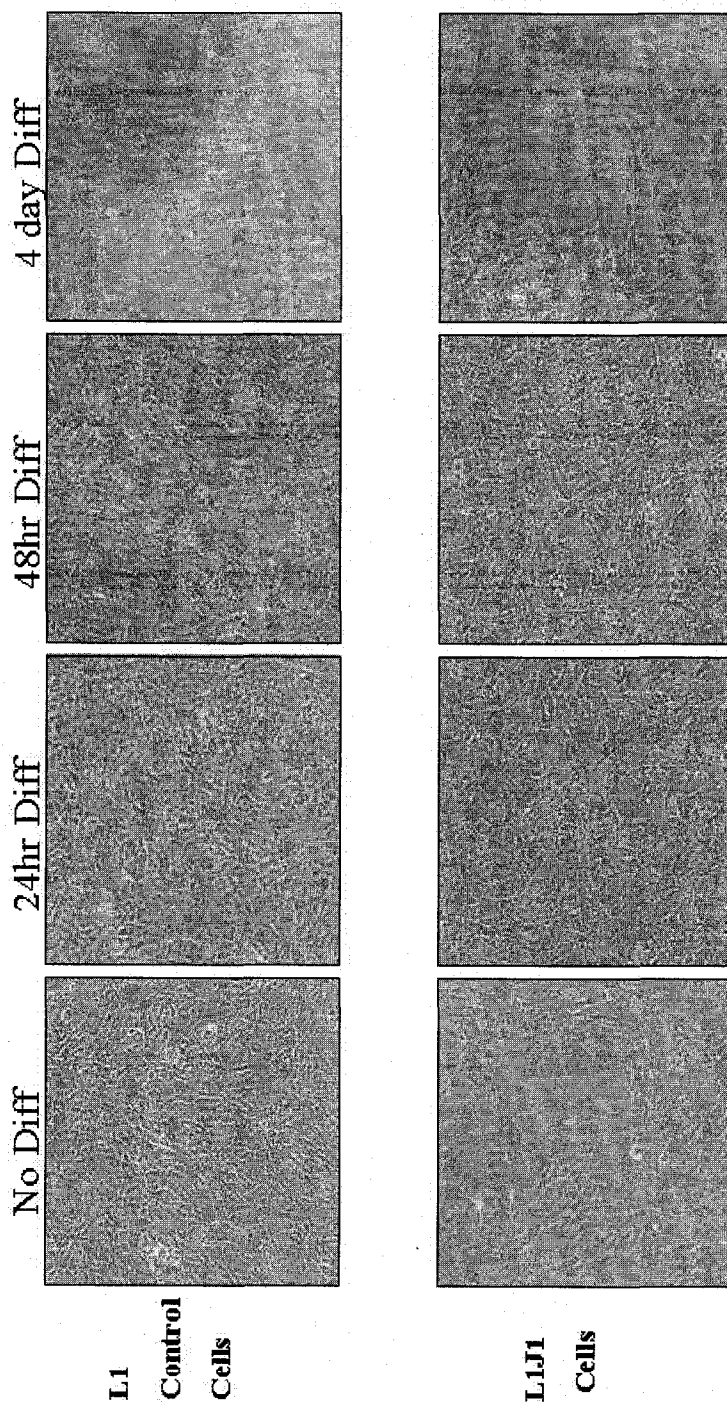


Figure 4. Constitutive expression of Jagged-1 changes the growth dynamics of L1 preadipocytes. A.) The above graph represents the survival rates of L1 control (represented by blue triangles) and L1J1 (represented by pink squares) cells grown in growth media. Cells were initially plated at a concentration of 1,000cells/ well and were collected for quantification after 24 hours, 48 hours, 4 days, 10 days, and 2 weeks of growth. Survival curves were based on the average total of three experiments. Survival rates of L1 control and L1J1 cultures were determined by counting adherent cells by trypsinization and determining cell numbers by using a hemocytometer. Error bars represent \pm standard error of mean. B.) The above graph represents the survival rates of L1 control (represented by blue triangles) and L1J1 (represented by pink squares) cells exposed to condition promoting differentiation (exposure to 48 hours of differentiation media followed by up to 2 weeks in maintenance media). Differentiation media was added to cultures after they reached 70% confluency. Cells were exposed to conditions promoting differentiation and were collected by trypsinization after 48 hours, 4 days, 10 days, or 2 weeks of exposure to these conditions. Survival curves were based on the average of three experiments. Cell numbers were determined by use of a hemocytometer. Error bars represent \pm standard error of mean.

Constitutive Expression of Jagged-1 Alters Lipid Accumulation - A Hallmark of a Mature Adipocyte.

In order to determine if constitutive expression of Jagged-1 changes the ability of L1 preadipocytes to differentiate, we stained L1 control and L1J1 cultures with Oil-red-O to visualize neutral lipid accumulation under growth and differentiation conditions. As expected, Oil-red-O staining was more intense in L1 control than in L1J1 cultures following exposure to differentiation media (Figure 6A). L1 control cultures also had more intense staining overall than did the L1J1 transfectants after every collection point (Figure 5A). In addition, L1 control cultures had a number of cells with droplets of lipid that were larger than the majority of droplets seen in L1J1 cultures (Figure 5B). These results indicate that constitutive expression of Jagged-1 causes the accumulation of lipid to be delayed and less extensive in this cell line.



(Figure 5 continued on top of next page)

B.

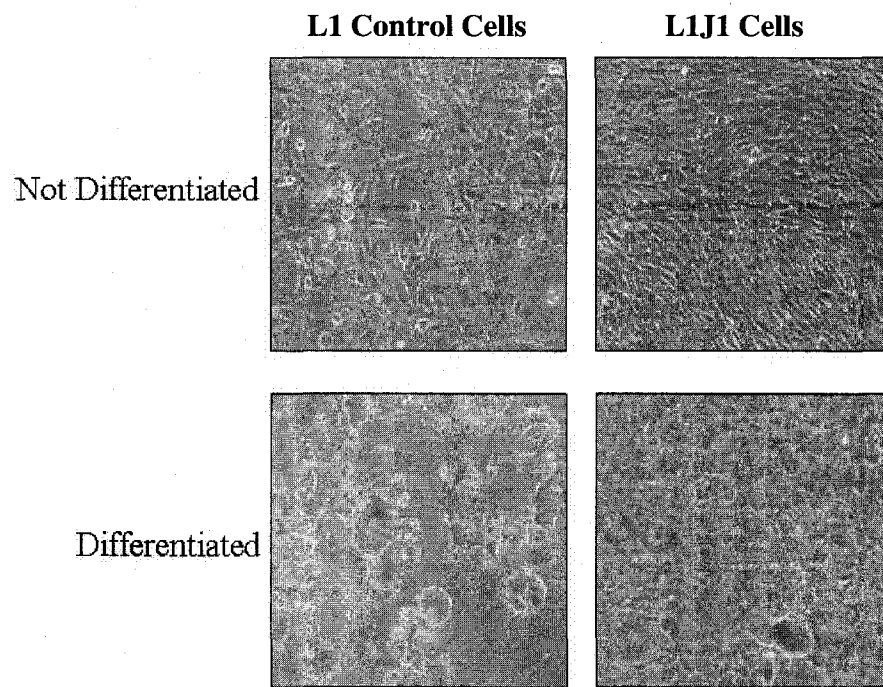


Figure 5. After exposure to differentiation conditions, lipid uptake is decreased in preadipocytes constitutively expressing Jagged-1. A.) Control preadipocytes and preadipocytes with constitutive Jagged-1 expression were grown in culture under growth conditions. When cultures were 70% confluent, growth media was changed to differentiation media. Cultures were grown under differentiation media for 24 hours, 48 hours, or 4 days and at these time points they were formalin fixed and stained with Oil-red-O to observe lipid accumulation. Micrographs, as seen above, were taken at 100x for analysis. B.) The bottom images are comparison of L1 control and L1J1 cells grown under growth or differentiation conditions. When cultures were 70% confluent, growth media was changed to differentiation media in one dish of each transfectant, and in the other dish was changed to fresh growth media. After two weeks cultures were formalin fixed and stained with Oil-red-O to observe lipid accumulation. Micrographs were taken at 200x for analysis.

Constitutive Expression of Jagged-1 Alters the Expression of Adipocyte-specific mRNA.

In order to determine if the reduction in Oil-red-O staining in L1J1 cells was due to an absence of adipocyte-specific gene expression, we examined these cells for expression of mRNA encoding genes specific for adipocyte differentiation (Table 3 in Materials and Methods). mRNA from L1 control and L1J1 transfectants were isolated from cells exposed to growth or differentiation media over a seven day period, as described in the Materials and Methods. Analysis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to qualitatively evaluate gene expression. RT-PCR revealed that the master adipogenesis transcription factor PPAR γ was expressed at later time points in L1J1 samples than it was in the L1 control samples (Figure 6). Since the transcription factor PPAR γ regulates FABP4 expression, we also examined cells for mRNA representing this gene product. Similar to the pattern of PPAR γ expression, FABP4 was expressed at later time points in L1J1 than in control L1 cells (Figure 6). FABP4 was expressed in L1 control cells within 48 hours of exposure to differentiation media, whereas FABP4 was not evident in L1J1 cell until four days after exposure (Figure 6). These results indicate that the delay in lipid uptake observed in the L1J1 transfectants is likely to be caused by a lag in differentiation. Interestingly, expression of the fatty acid metabolizing enzyme Lipoprotein Lipase (LPL) displays the same pattern of expression in both L1 control and L1J1 transfectants. Since expression of LPL is not solely dependent on PPAR γ activity (Mead, 2002), it is likely that constitutive expression of

Jagged-1 influences some, but not all of the mechanisms promoting differentiation in these cells.

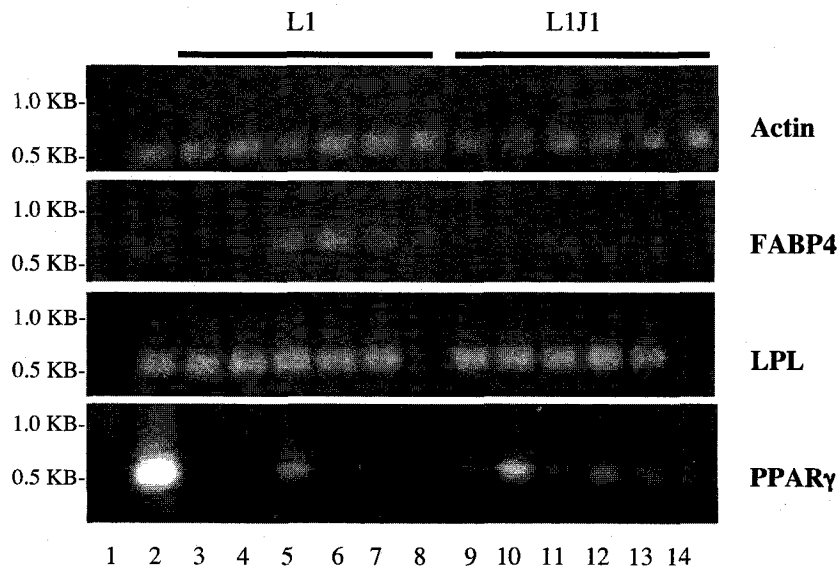


Figure 6. The expression of adipocyte-specific genes are delayed in L1J1 cells. PCR amplification was used to determine the presence of adipocyte-specific genes in cDNA synthesized from mRNA collected from L1 control and L1J1 cultures. The above image is a representative of triplicate PCR reactions performed to look for genes of interest. Genes of interest included β -actin (control), PPAR γ , FABP4, and LPL and were amplified using primers listed in Table 3. mRNA was isolated from samples at various time points during exposure to conditions promoting differentiation. Samples represented above are: 1) negative control (primers only), 2) positive control cDNA (mouse adipose tissue), 3) L1 control cells grown in differentiation media for 24 hours, 4) L1 control cells grown in differentiation media for 48 hours, 5) L1 control cells grown in differentiation media for 7 days, 6) L1 control cells grown in differentiation media for 10 days, 7) L1 control cells grown in differentiation media for 2 weeks, 8) L1 control cells grown in growth media for 48 hours, 9) L1J1 cells grown in differentiation media for 24 hours, 10) L1J1 cells grown in differentiation media for 48 hours, 11) L1J1 cells grown in differentiation media for 7 days, 12) L1J1 cells grown in differentiation media for 10 days, 13) L1J1 cells grown in differentiation media for 2 weeks, 14) L1J1 cells grown in growth media for 48 hours.

Constitutive Expression of Jagged-1 Delays Expression of Adipocyte-specific Proteins in L1J1 Cells.

Expression of mRNA levels does not necessarily reflect the expression of their encoded proteins. Thus, we also characterized PPAR γ and FABP4 protein expression by western blot analysis of cell lysates isolated from L1 control and L1J1 cell cultures exposed to growth and differentiation conditions. Qualitative comparison of the two cell lines indicated that much like the mRNA expression profile, PPAR γ protein expression is delayed in L1J1 cell lines as compared to L1 control lines (Figure 7). As expected, L1 control cells express PPAR γ 48 hours after exposure to differentiation conditions, but was not detectable in the L1J1 transfectants until after seven days exposure. More PPAR γ expression was observed in L1 control cells than L1J1 cells at both the 48 hours and 7 day time periods. Interestingly a second PPAR γ specific band was observed in L1 control protein but not L1J1 protein lysates. This band may reflect PPAR γ 1 isoform which is smaller than PPAR γ 2, but the identity and significance of this additional band is unclear at this time. These results indicate that increased Jagged-1 expression affects PPAR γ protein expression.

Qualitative analysis of FABP4 expression indicates that expression of this protein is decreased in L1J1 cells compared to L1 control cells following exposure to differentiation media (Figure 8). FABP4 protein expression was observed in protein lysates isolated from L1 control and

L1J1 cells grown under differentiation conditions. Ponceau staining indicated that loading of protein was equal (data not shown); however FABP4 expression in the L1J1 pools was fainter than expression in L1 control pools. These results indicate constitutive Jagged-1 expression decreases the expression of FABP4, most likely due to the delay in PPAR γ expression.

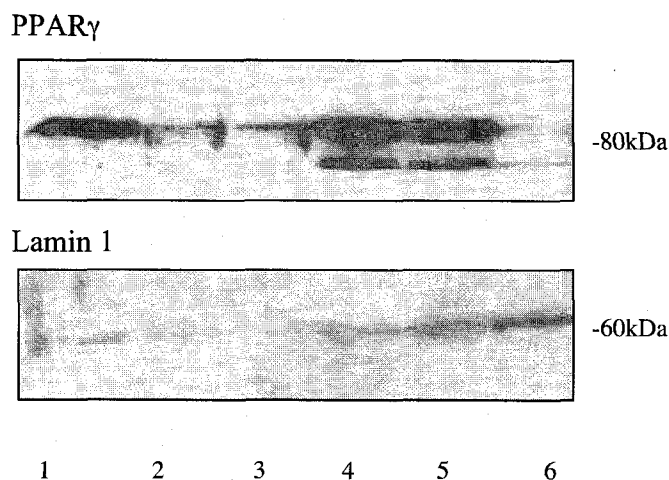


Figure 7. Expression of PPAR γ protein is delayed in cells that over-express Jagged-1. The top image is a representation of an immunoblot for PPAR γ (performed in triplicates) on nuclear protein isolated from L1 control and L1J1 cell lysates. The bottom image is a blot to detect Lamin B1 expression, which was a loading control for the PPAR γ blots. Lanes labeled above represent protein isolated from the following samples: 1) L1J1 cells grown in differentiation media for 7 days, 2) L1J1 cells grown in differentiation media for 48 hours, 3) L1J1 cells grown in growth media for 48 hours, 4) L1 control cells grown in differentiation media for 7 days, 5) L1 control cells grown in differentiation media for 48 hours, 6) L1 control cells grown in growth media for 48 hours.

FABP4

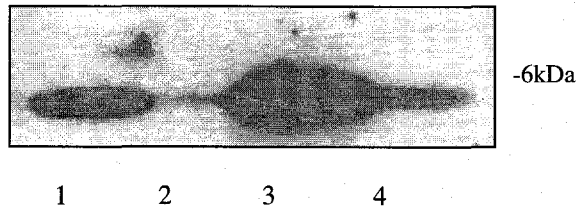


Figure 8. FABP4 protein expression is greater in control cells than cells with constitutive expression of Jagged-1. The above image represents one of two FABP4 blots performed on cytoplasmic protein isolated from L1 control and L1J1 cell lysates. Lanes labeled above represent pools of the following samples: 1) L1 control cells prior exposure to differentiation media, 2) L1 control cells after exposure to differentiation media, 3) L1J1 cells prior exposure to differentiation media, 4) L1J1 cells after exposure to differentiation media.

Characterization of L1 Preadipocyte-derived Fat Pads in Athymic Nude Mice.

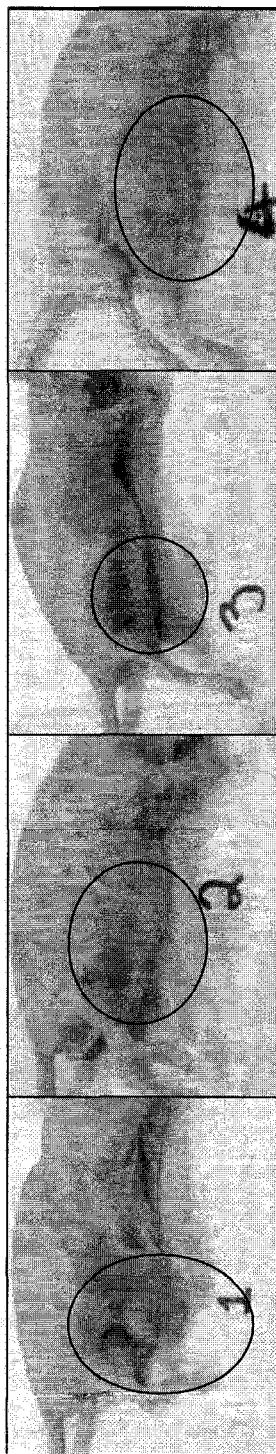
L1 Control and L1J1 Cells form Fat Pads when Subcutaneously Injected into Athymic Nude Mice.

In culture, we observed a delay in preadipocyte differentiation in 3T3-L1 preadipocytes with constitutive expression of Jagged-1 and therefore wanted to determine the effect that constitutive expression of Jagged-1 had on preadipocyte differentiation in an *in vivo* model of adipogenesis. L1 control preadipocytes form fat pads *in vivo* when they are subcutaneously injected into athymic nude mice (Kawaguchi, 1998). Therefore, we used this model to determine the effects that constitutive expression of Jagged-1 had on fat pads that develop from injected cell lines. To begin, we made subcutaneous injections of either 500,000 L1 control or L1J1 cells into eight athymic nude mice. To promote fat pad development, cells were also mixed with basement membrane extract (Matrigel, B&D) that contained the full complement of growth factors or a Matrigel preparation in which growth factors were partially removed. In the second study, 250,000 cells from each cell line were mixed with Matrigel with reduced growth factors only and injected into six mice. In both trials, fat pads began to develop in all the nude mice one to two weeks after injections. Unfortunately, in both experiments tumors also formed at the site of injection that quickly obscured the developing fat pads (Figure 9). Measurements of the tumors indicated that there did not appear to be any correlation between tumor size and the cell line that was

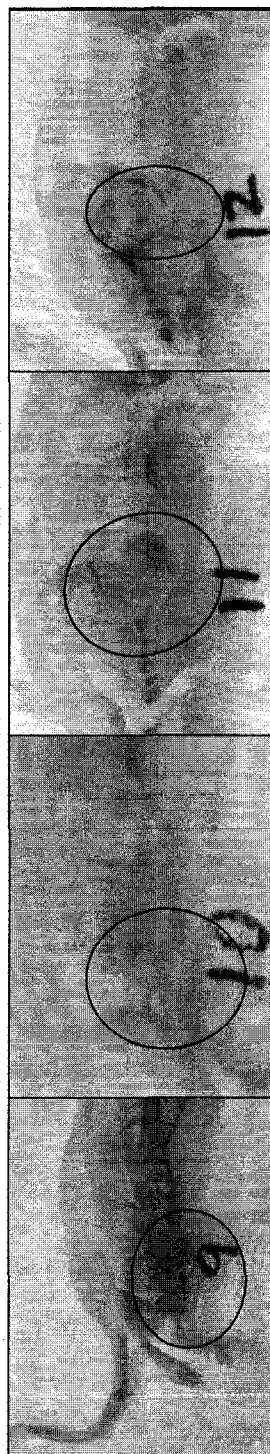
injected (Data not shown). However despite tumor formation, fat pads still developed four weeks after initial injections as evident from analysis of dissected tissue (Figure 9B).

A.

Mice injected with L1 control cells



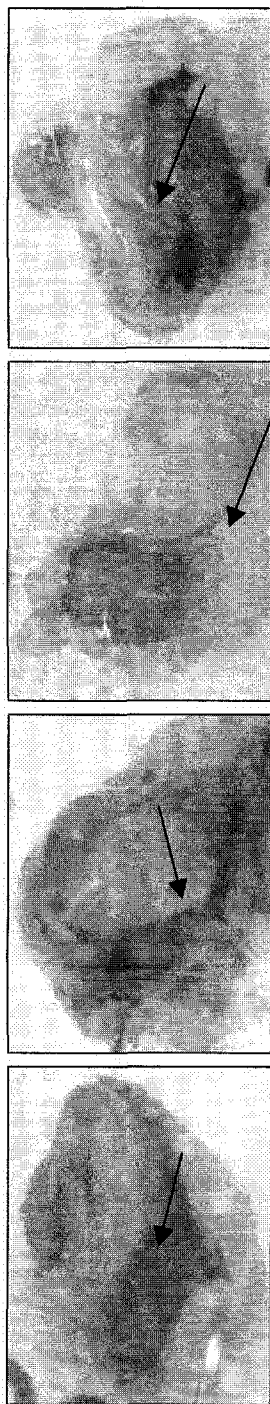
Mice injected with L1J1 cells



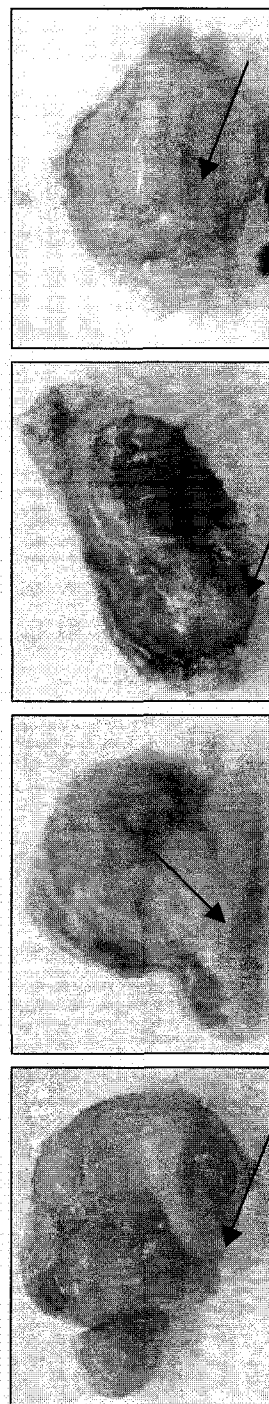
(Figure 9 continued on the top of the next page).

B.

L1 control derived tumors



L1J1 derived tumors



(Figure 9 continued on the top of the next page)

L1 Control-derived Fat Pad

L1J1-derived Fat Pad

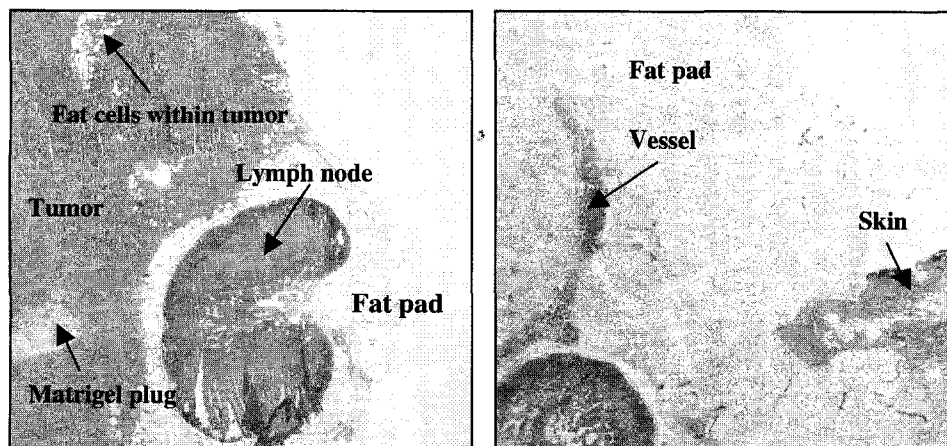


Figure 9. Fat pad and tumors developed in athymic nude mice after subcutaneous injection of L1 control and L1J1 preadipocytes. A.) Above images are representative of eight out of twelve fat pads and tumors that formed in nude mice from the second study three weeks after injection of L1 control or L1J1 cells. Tumor and fat pad growth is circled in above images. B.) Representative images of dissected fat pads and tumors from the second experiment three weeks after injection of L1 and L1J1 preadipocytes. Arrows indicate the fat pad that formed at the site of injection with the surrounding tissue primarily composed of tumor. The top row represents fat pads and tumors dissected from mice injected with L1 control cells and the bottom images are from mice injected with L1J1 cells. C.) Above micrographs were taken at 4x for visualization of entire fat pad. As indicated, many sections contained lymph nodes, skin, and muscle tissue around or within the fat pad. Many fat pad sections also contain large areas of tumor growth, which is seen as areas of dark purple in above images.

Prior to the euthanization of mice, fat pads and tumors were monitored in the living animals using Magnetic Resonance Imaging (MRI) at two and three weeks after injections of cells (Figure 10). MRI spectrophotography images of mice confirmed both tumor and fat tissue development at the site of injection (Figure 10). In these images, adipose tissue was observed as areas of white in spectrophotographs. Due to human error, measurements of individual fat pad size were not taken during MRI analysis. In addition, tumors developed prior to the first MRI procedure and therefore do not provide data related to tumor versus fat pad development. None-the-less, these images demonstrate that small fat pads did develop at injection sites.

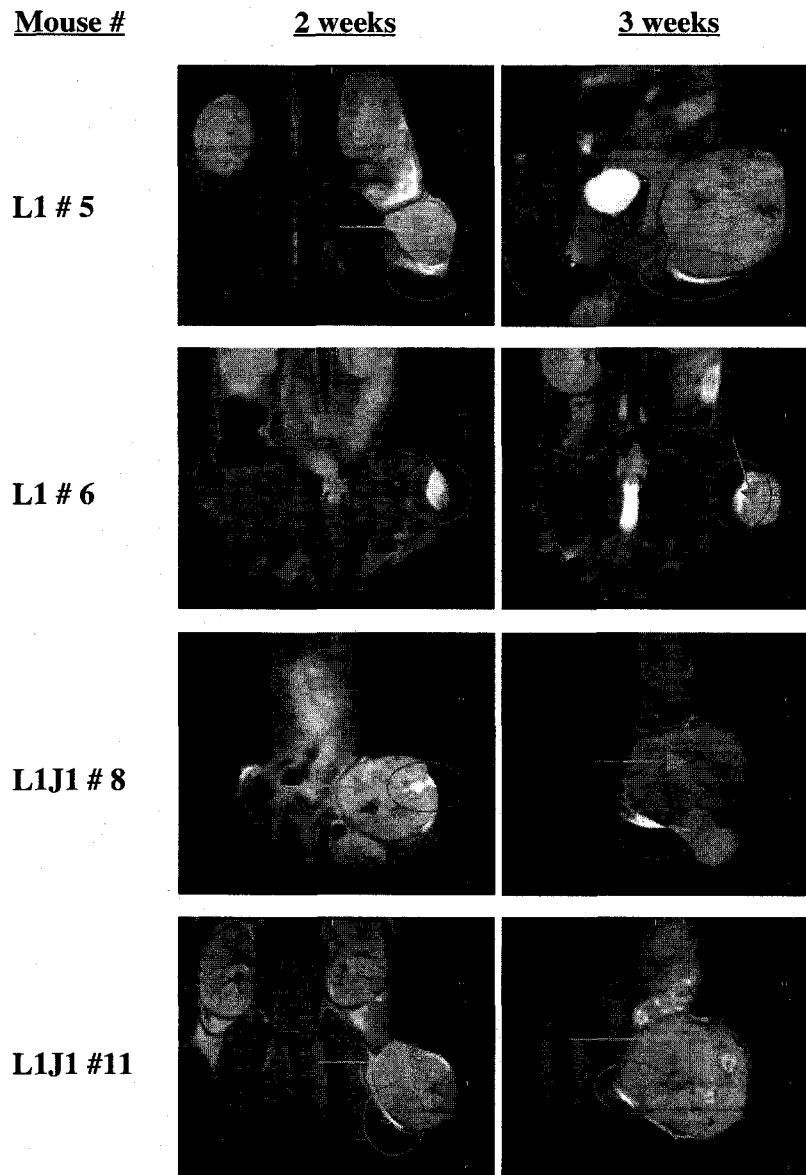
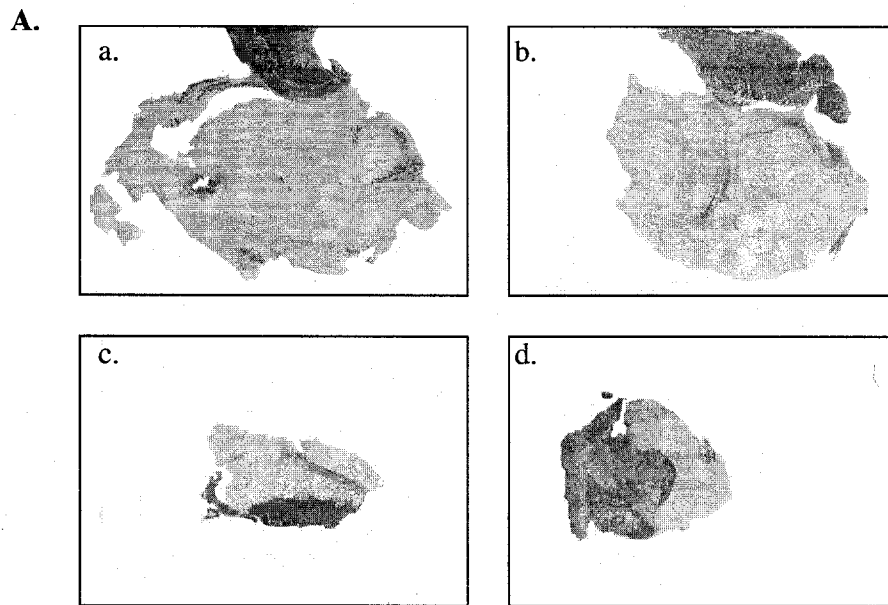


Figure 10. Both fat pads and tumors developed in athymic nude mice following injection of preadipocytes. MRIs were performed on mice to observe the formation of fat pads and tumors two and three weeks after injection of L1 control and L1J1 cells. MRIs were performed on all mice belonging to the second study group. The above images represent MRIs from two mice that were injected with either L1 control or L1J1 cells two weeks after introduction of cell lines. Adipose tissue appears as the areas of white (in red circles), as confirmed through comparison to epididimal tissue (not shown), which is known to contain adipose tissue. Tumor masses are solid areas represented by a green arrow can also be seen in most mice two weeks after injections and are located adjacent to fat pads.

Fat Pads that Developed from L1J1 Preadipocytes are Smaller than those that are Derived from L1 Control Cells.

In order to evaluate the overall structure of the fat tissue, sections of fat pads were prepared for histological analysis using Hemotoxylin and Eosin (H & E), or Mason Trichrome stains, as described in Materials and Methods. We measured the total circumference of the fat pad in 5 μ m H & E stained serial sections (Figure 11A). Averages for each fat pad were determined from measuring the circumference of three sections of each fat pad using Scion Image, and these values were used to determine the average fat pad size for L1 control and L1J1 fat pads. Using this method, we found that the average fat pad area was significantly larger ($p < 0.01$) in the L1 control than in the L1J1-derived fat pads (Figure 11B). These results suggest that constitutive expression of Jagged-1 in preadipocytes either inhibits or delays adipose tissue development *in vivo*.



B.

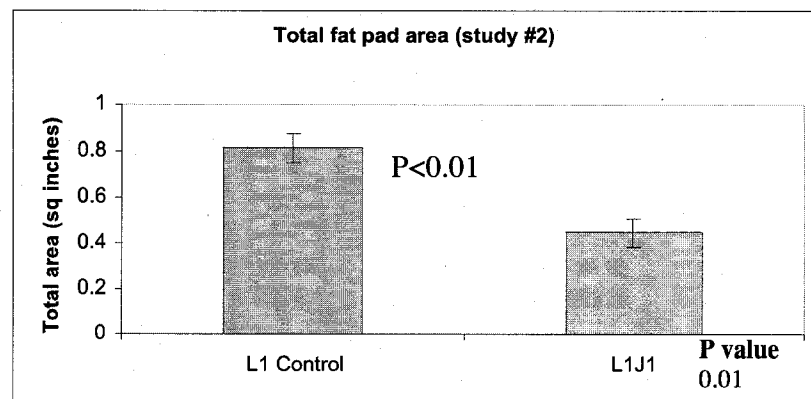
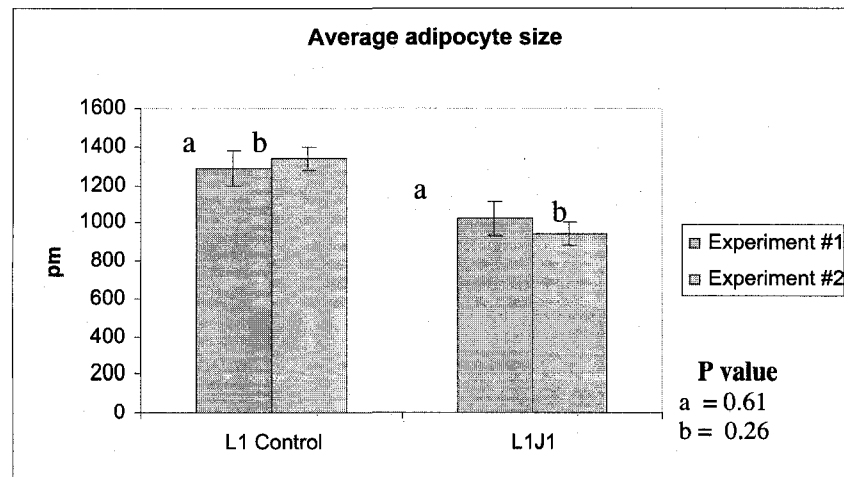


Figure 11. Fat pads derived from L1J1 transfectants are significantly smaller than L1 control-derived fat pads. A.) Micrographs of 5 μ m sections of L1 control and L1J1 derived fats pads were taken from mice in group 2 at 8x magnification on a dissecting scope. Sections were stained with Mason-Trichrome for visualization of connective tissue structure. Images above are two representatives of sections from L1 control derived pads (a. and b.), and L1J1 derived fat pads (c. and d.) from the second experiment. Black arrows point to fat pad area between L1 control and L1J1-derived tissues. B.) The above graphic shows the average differences between the total area of L1 control and L1J1-derived fat pads. Bar graphs represent the average fat pad area for 5 μ m sections as determined by averaging the calculated circumference of each section. Evaluations were done on the second experiment only. Error bars represent \pm standard error of mean.

In order to determine if smaller fat pad size was due to a decrease in the overall size of mature adipocytes, we looked at the differences between average adipocyte size in each fat pad. Analysis was performed using Spot Imaging by measuring the circumference of each cell within a 2,000 μ m area. Adipocytes in L1 control-derived fat pads averaged 1,290pm SE \pm 1.04 in first experiment, and 1,021pm SE \pm 0.05 in the second experiment. Adipocytes from L1J1-derived fat pads averaged 1,335pm SE \pm 1.04 in the first experiment, and 942pm SE \pm 0.05 in the second experiment. Quantitative analysis of adipocytes in L1 control and L1J1-derived fat pad determined that there was no significance difference between adipocyte size in fat pads (p=0.61 experiment one, p=0.26 experiment two), although average size trended downward (Figure 12).

Since fat pad size could also be due to differences in the number of fat cells per area, we counted average number of cells/ area in several sections of fat pads. To do this, we counted the number of adipocytes within a 2,000 μ m area of tissue for each fat pad as with average fat cell size. No statistical difference was seen between the number of adipocytes in the given areas for each fat pad (Figure 12). However, L1 control-derived fat pads had, on average, did contain fewer adipocytes than L1J1-derived fat pads (Figure 12).

A.



B.

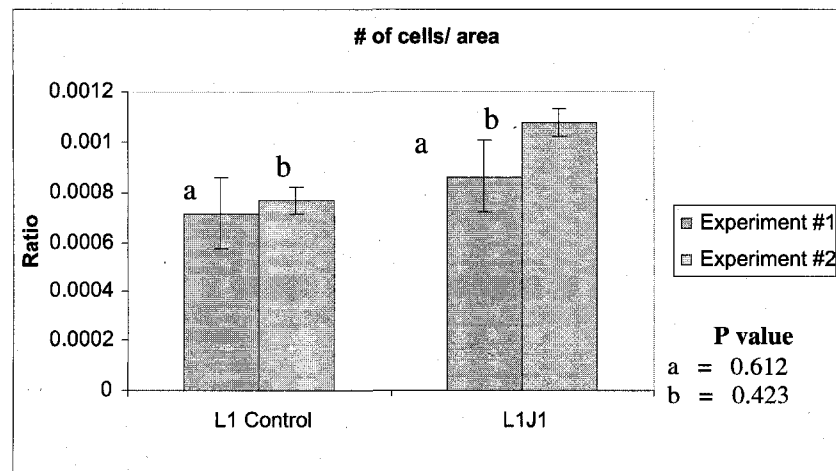
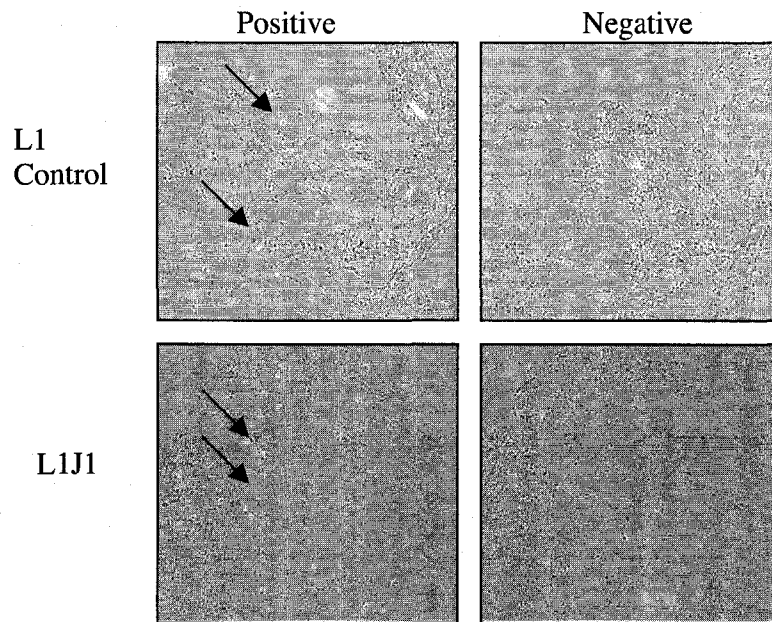


Figure 12. No statistical difference was found between the average cell number or between adipocytes size in L1 control and L1J1-derived fat pads. A.) The above image is a graphic analysis of the average cell number within each type of fat pad. Average cell numbers were determined by counting the number of adipocytes within three given areas. These cell numbers were averaged together to determine the average number of adipocytes for each individual fat pad. Average fat cell number for experiment #1 and experiment #2 is represented as blue and purple bars, respectively. B.) Cell size was determined by measuring the circumference of individual adipocytes within 2,000 μ m area of fat pad. The average adipocyte size was determined for each fat pad and these values were then combined to determine the average adipocyte size per fat pad type. The image above is a graphic evaluation of average adipocyte size in L1-derived fat pads and L1J1-derived fat pads from experiment #1 (blue columns) and experiment #2 (purple columns).

Differences in Levels of Proliferating Cells are not seen Between Fat Pads Derived from L1 Control L1J1 Preadipocytes.

To determine if fat pad size reflected the number of proliferating cells within the fat pad, we used BrDu to tag replicating cells. BrDu incorporates itself into the DNA of replicating cells, and therefore cells that take up BrDu represent proliferating populations. For this procedure, mice were injected twice with BrDu at 24 hours and two hours prior to euthanization. 5 μ m sections were stained against BrDu, as described in Materials and Methods. Analysis of these slides found few areas of positive cells within either group of fat pads. Whether BrDu positive cells were preadipocytes, tumor cells or leukocytes is unclear as pathological examination did not confirm cellular identity. These results indicate that at the time of euthanization, preadipocyte populations within the adipose tissue were not replicating at an appreciable rate.

A.



B.

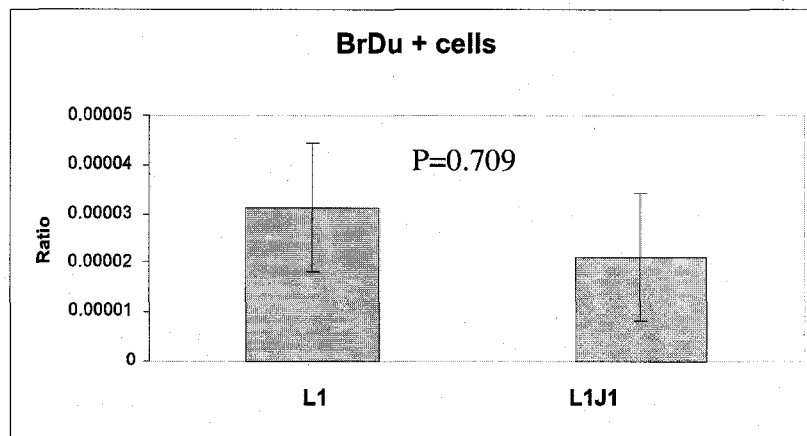


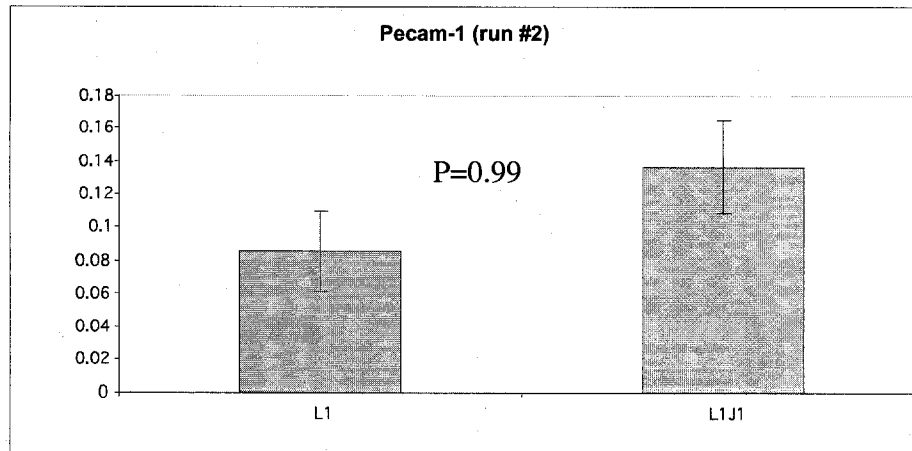
Figure 13. Few proliferating cells were found in L1 control and L1J1-derived fat pads. 24 hours and 2 hours prior to being euthanatized, mice were injected with BrDu. BrDu tags proliferating cells, allowing them to be visualized when stained with an anti-BrDu antibody. A.) The above images represent 5 μ m sections from L1 control and L1J1-derived fat pads that were stained against BrDu. Negative BrDu stain represents sections stained with secondary antibody, only to rule out any background expression. B.) The above graphic represents analysis of BrDu positive cells found within a given 200x field of vision. No significant differences between numbers of proliferating cells were seen within L1 control and L1J1-derived fat pads.

L1 Control and L1J1-derived Fat Pads Display Differences in Morphology and Vascular Network.

Since vascular development has been found to be correlated with adipose tissue development, we examined fat pad sections for vessel structure. Sections of 5 μ m fat pads were stained with anti-PECAM-1 to visualize endothelial cells, which are the cells that line blood vessels (see Appendix D.). PECAM-1 positive cells take on an orange color after staining, allowing positive areas to be quantified using Scion imaging as described in Materials and Methods. However, due to the large amount of background, fat pad sections in the second run of the experiment were also stained with Mason-Trichrome to help better visualize collagen and vessel structure (Figure 15A).

Quantification of PECAM-1 positive stained cells in L1 control and L1J1 derived fat cells did not determine any correlation between vessel area and type of injected cells (Figure 15B). However, morphological observations of L1J1-derived fat pads revealed that vessels were dilated with fewer numbers on branches, whereas vessels in L1 control-derived fat pads were smaller and contained a number of budding vessels (Figure 15A). In addition L1J1-derived fat pads contained larger areas of necrotic tissue. Together, these results suggests that the in the L1J1, fat pad size may be due to increased cell death from a less developed vascular network.

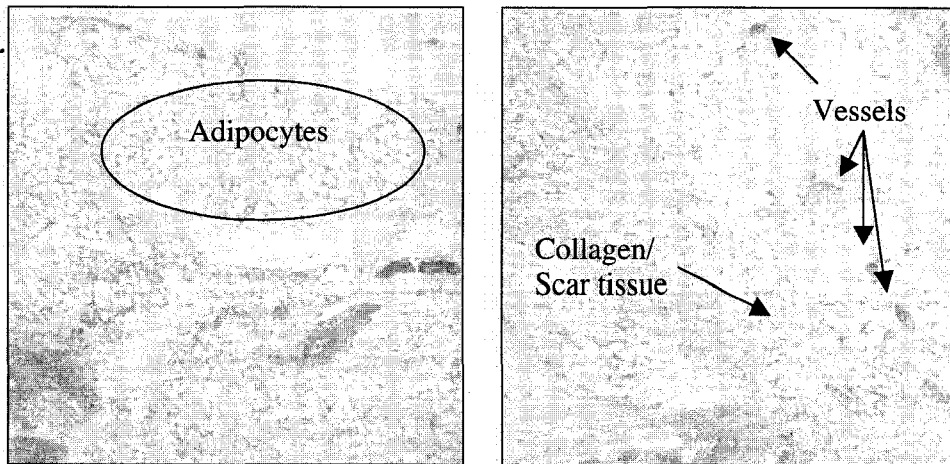
A.



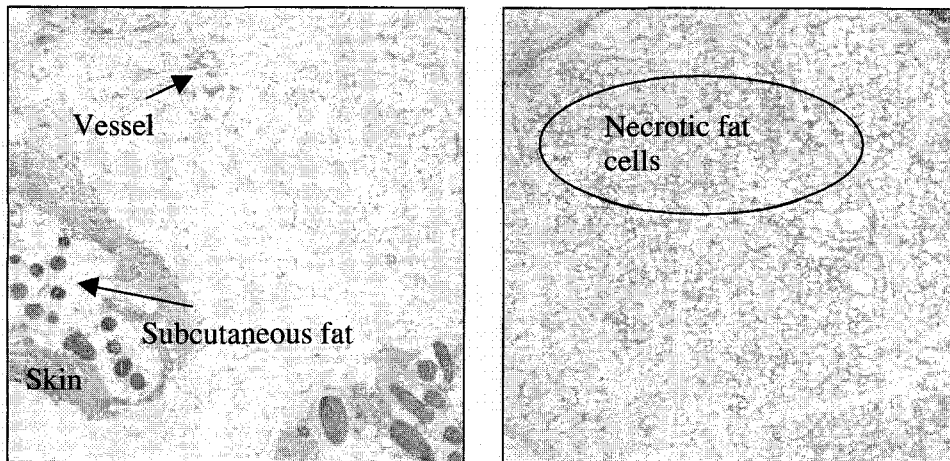
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B.

L1 Control-derived Fat Pads



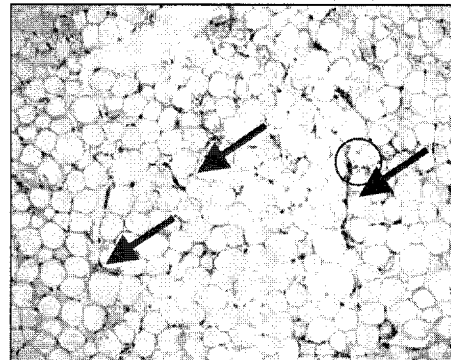
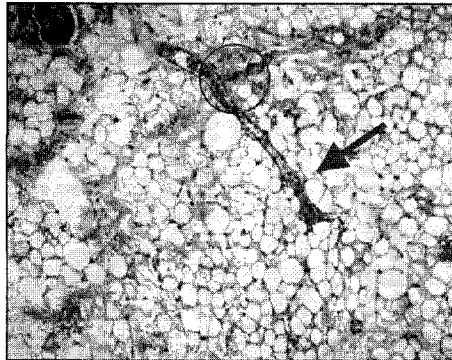
L1J1 -derived Fat Pads



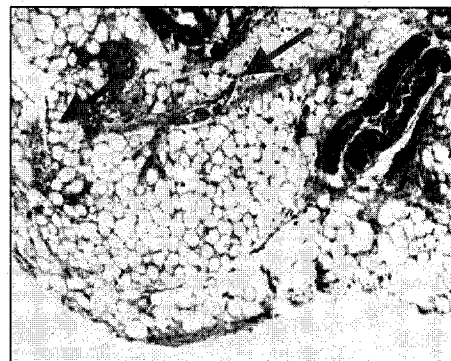
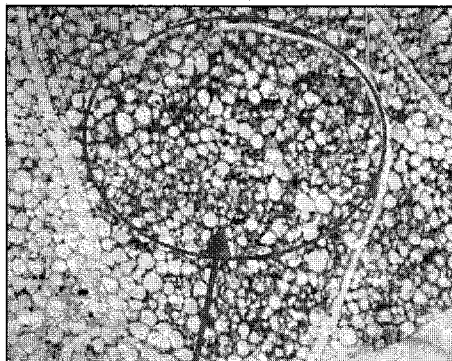
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C.

L1 Control-derived Fat Pads



L1J1-derived Fat Pads

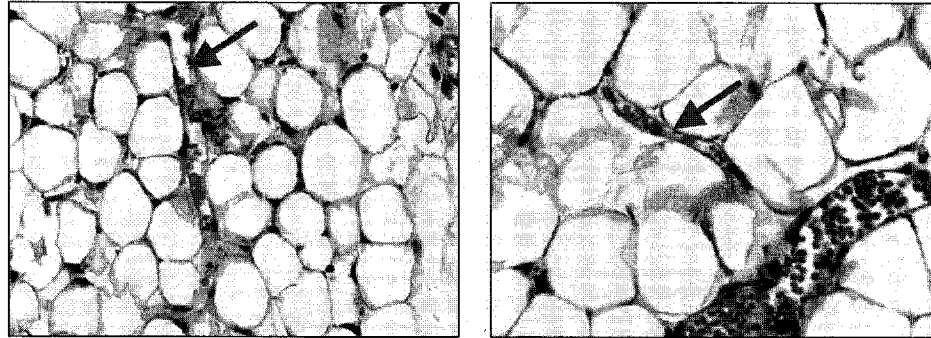


Necrosis

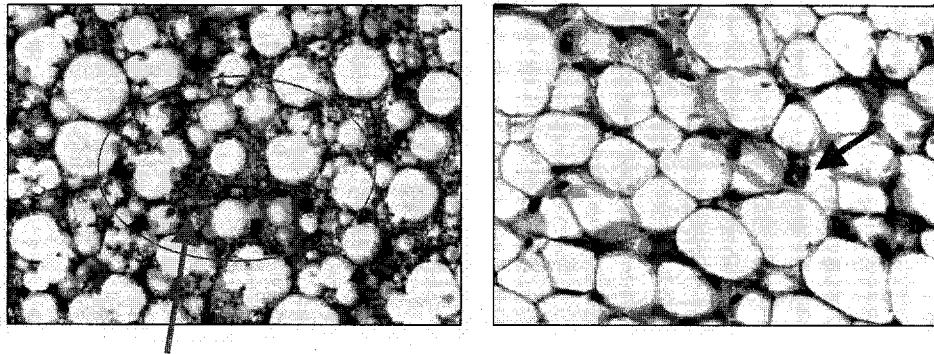
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D.

L1 Control-derived Fat Pads



L1J1-derived Fat Pads



Necrosis

Figure 14. Fat pads derived from L1 control cells appear to have greater vessel area. A.) 5 μ m sections of fat pad were stained with PECAM-1. Areas with positive PECAM-1 stain were quantified using Scion imaging. Above graph is a comparison of average vessel area within each type of fat pad. B.) Above images are 5 μ m sections taken at 200x magnification of L1 control and L1J1-derived fat pads that were stained with Mason Trichrome to help visualize vessel structure. Areas of vessels are indicated above by blue arrows. Sections were also observed for areas of budding vessels, which are in blue circles in above images. C.) Above images are 5 μ m sections taken at 400x magnification of L1 control and L1J1-derived fat pads that were stained with Mason Trichrome to help visualize vessel structure. Areas of vessels are indicated above by blue arrows. D.) 5 μ m sections of fat pads were stained with hemotoxylin and eosin for visualization of fat pad structure. For observation of fat pad morphology, micrographs were taken at 100x. Many of these images contained vessels, subcutaneous fat, skin, and collagen. In these images many areas of fat pads had what appeared to be necrotic areas, as

indicated.

CHAPTER IV

DISCUSSION

The process of adipogenesis requires coordination of many signaling pathways including the Notch system. To date, the mechanism(s) by which Notch regulates adipogenesis is unclear. Furthermore, the contribution of specific Notch ligands in the regulation of adipogenesis is unknown. To address this issue, we examined the influence that the Notch ligand Jagged-1 had on adipogenesis and adipose tissue development. Our research with L1 preadipocytes constitutively expressing Jagged-1 yielded results similar to other studies that examined the effect of Notch-1 activation on adipogenesis. In summary, we found that inappropriate expression of Jagged-1 impairs adipogenesis *in vitro* and adipose tissue development *in vivo*.

Since proliferation is the first phase of final differentiation, we studied survival curves of L1 control and L1J1 cell lines (Figure 4). We found that constitutive Jagged-1 expression decreased cell proliferation, *in vitro* (Figure 4B). Previous research has determined that fibroblast cell growth is decreased when Jagged-1 is over-expressed or when Notch-1 is constitutively active (Nicolas, 2003, Small, 2003). In contrast, Notch activation can increase cell proliferation, as seen proliferation of hematopoietic stem cells in blood (Karanu, 2000). Similarly, Jagged-1 mediated Notch signaling has is associated with increased cell proliferation in bone marrow cells (Li, 1998). Thus, Jagged-1/ Notch control

of cell proliferation is context dependent, and in our 3T3-L1 preadipocytes, inappropriate Jagged-1 expression appears to cause growth suppression.

Conversely, no differences were found between the numbers of proliferating cells *in vivo*, as determined by BrDu staining of fat pad sections. Indeed, very few BrDu positive cells were identified in fat pads-derived from either L1 control or L1J1 transfectants. This was surprising because preadipocytes have been determined to proliferate *in vivo* when stimulated (Fukumura, 2003), and transplanted autographs of adipose tissue into mice have been demonstrated to promote proliferation of endogenous preadipocytes in surrounding tissue (Kawaguchi, 1998). Since adipose tissue did develop from these cells, it is likely that the injected preadipocytes proliferated at earlier time points and that this process had ceased before the fat pads were processed. It is possible that the decreased size of the fat pads derived from L1J1 transfects was due to Jagged-1 suppression of preadipocyte proliferation; however, we do not have any *in vivo* evidence to support this hypothesis at this time.

Lipid accumulation in proliferating preadipocytes *in vitro* and *in vivo* is promoted when cells are exposed to conditions that promote final differentiation. Our qualitative observations of L1 control and L1J1 cells exposed to differentiation conditions *in vitro* indicated that lipid accumulation was greater in L1 control than in L1J1 cells indicating that adipogenesis was suppressed in these cells. Studies by both Garces et al., 1997 and Ross et al. 2004, individually support a role for Notch as both a positive and negative regulator of adipogenesis, respectively. While seemingly contradictory, the studies collectively reflect the

need for precisely timed Notch activation in order for adipogenesis to be completed. The partial suppression of adipogenesis in L1J1 transfectants may reflect only a modest change in normal Notch signaling in these cells. Interestingly, constitutive expression of the active form of Notch-1 both decreased and abrogated PPAR γ expression (Ross, 2004), whereas in our study, constitutive expression of Jagged-1 only delayed PPAR γ expression. These results suggest that constitutive expression of this ligand cannot maintain full activation of the receptor. Notch-ligand interactions are regulated by post-translational modification, such as glycosylation, and it is possible that Jagged-1 activity is partially down-regulated by this mechanism. Therefore, when the ligand is expressed at higher than normal levels it may be down-regulated by post translational modifications such as glycosylation which suppresses Jagged-1/Notch interactions (Yang, 2005).

As previously indicated, the decreased lipid accumulation within L1J1 cells is likely due to the delay in PPAR γ expression. PPAR γ is a master regulator of lipid accumulation; and inhibition of PPAR γ expression can prevent lipid accumulation. Thus a delay in PPAR γ expression would affect consequence downstream factors (Zuo, 2006). Similar to our findings, observations by Liu et al. 2004, found that when PPAR γ expression is inhibited, FABP4 expression is also suppressed and adipogenesis does not occur. FABP4 facilitates fatty acid diffusion within the adipocytes. Without FABP4, lipid can not adequately accumulate (Maeda, 2003). Of interest to the current study was also the observation of two PPAR γ positive bands identified by western blot analysis.

PPAR γ 2 is currently the main member of the PPAR family involved in adipogenesis (Tontonoz, 1994). PPAR γ 1 is also expressed in adipocytes but there is some evidence indicating it is a less potent activator of adipogenesis than PPAR γ 2 (Mueller, 2002). Our PPAR γ antibody was not specifically designed to detect only PPAR γ 2 and the second band seen in the study could represent PPAR γ 1. Since L1J1 cells did not express the lower PPAR γ positive bands, it is possible that both isoforms are required to activate FABP4 expression in L1 preadipocytes. Together, these data indicate that the constitutive expression of Jagged-1 interferes with adipogenesis by affecting the status of PPAR γ expression.

Expression of Pref-1, a preadipocyte marker, was also detected in L1 control and L1J1 cell cultures. However, Pref-1 protein was expressed at later time points in L1J1 samples than in L1 control cells exposed to differentiation media (data not shown). Since Pref-1 inhibits adipogenesis, the expression of Pref-1 at later time points in our L1J1 cell culture indicates a delay in the down-regulation of the protein that may contribute to the decreased differentiation potential of these cultures (Smas, 1999; Boney, 1996). This evidence is significant, because Ross et al. 2006 suggested Hes-1, a known target for Notch signaling, regulates adipogenesis through the promotion of Pref-1 expression. In our cells, Jagged-1/ Notch signaling inhibits the complete down regulation of Pref-1 expression thus suppressing adipocyte development.

Another factor important for adipose tissue development is angiogenesis. In our fat pads no significance difference was seen in total vessel area between fat

pad types. However, qualitative analysis of vessel morphology indicates that L1J1 fat pads contained large, dead-ended vessels, whereas vessels in L1 control-derived fat pads were smaller in diameter and contained more branches, indicating a more extensive vascular network within these tissues (Figure A.16.). The presence of misshapen and fewer branching vessels in L1J1 adipose tissue would explain why many areas of necrosis were also noted within these fat pads. Increased vascular scaffolding has been previously determined to support adipose tissue development (Hemmrich, 2005) and when vessel development is inhibited *in vivo*, adipose tissue growth is also stunted (Yamaguchi, 2005). In our fat pads, a poor vascular network would reduce blood flow into the tissue that could result in necrosis. Therefore, the misshapen vessels that we observed in L1J1 fat pads could be one factor that caused a decrease in total fat pad size in L1J1 fat pads in our study.

The differences in vessels that developed in L1J1 fat pads could be a direct result of increased Jagged-1 expression. It is possible that constitutive expression of Jagged-1 is causing a deregulation of an angiogenic factor. VEGF is a mitogen for endothelial cells and promotes vascular branching (Neufeld, 1999). In L1 control cells VEGFA is expressed significantly more than in L1J1 transfectants *in vitro*, as determined by ELISA data (Small, data not shown). In previous research, increased expression of Notch ligands has been determined to inhibit VEGFA expression in endothelial cells (Williams, 2006). Thus, constitutive Jagged-1 expression in L1J1 cells might be inhibiting VEGFA expression and negatively affecting the vasculature of these fat pads.

Lastly, we recognize different limitations to this study. One major limitation was the development of tumors in the athymic nude mice. Since tumors were present at the time MRIs were taken, we were not able to determine if they developed before or after the fat pads. No evaluations of tumor sizes or rate of tumor growth were taken, but there appeared to be no correlation between final tumor size and the cell type that was injected. It is likely that tumors were caused by growth factors injected into the tissue. Growth factors rapidly promote proliferation of cells and would promote the proliferation of any immortalized cells within the area (Gregoire, 1991, Kanai-Azuma, 1993). Although we have observed that injection of Matrigel only does not promote tumorigenesis, we have found that tumors do form when injected with Matrigel and 3T3 fibroblasts. It is possible that the combination of these cells with Matrigel produces a tumorigenic response.

Another limitation to this study is that we cannot be certain that fat pads were truly derived from the injected cells. Fat pads could have developed from endogenous preadipocytes that were recruited to the area of injection. We attempted to solve this problem by transfecting our cells with GFP constructs to tag the injected cell population (Appendix A). However, results were not clear and we could not positively identify GFP population.

Lastly, it is important to recognize that we only observed the affect of one Notch ligand, Jagged-1, on preadipocyte differentiation. It is possible that different Notch ligands exert unique effects during this process as well. For example, in hematopoietic stem cells, Jagged-1 ligands promote cell growth

(Karanu, 2000), where as soluble forms of Delta-like-1 inhibited the differentiation of hematopoietic stem cells (Han, 2000). This data indicates that Notch ligands can have opposite effects on Notch signaling resulting in different cell fate decisions. Therefore, it is important to note that expression of other Notch ligands in 3T3-L1 preadipocytes could have promoted adipogenesis. Since other Notch ligands, including Jagged-2 and Delta-1 display disparate patterns of expression under growth and differentiation condition, future studies on the roles of both in adipogenesis and adipose tissue development may lead to insight into the nuances of the mechanism by which the Notch signaling system regulates this important physiological process.

In conclusion, the present study indicates that Jagged-1 is a regulator of preadipocyte proliferation and differentiation. We observed *in vitro* that constitutive Jagged-1 expression in 3T3-L1 preadipocytes prevents expression of adipocyte-specific markers. Our data indicates that constitutive expression of Jagged-1 has a similar effect on adipogenesis *in vivo* as it does *in vitro*. Differences in fat pads that formed as a result of injected cells may be the result of decreased preadipocyte proliferation and/ or incomplete differentiation of preadipocytes due to increased expression of Jagged-1. Protein and RNA isolated from cells grown in culture exhibit a delay in PPAR γ expression and a delay in FABP4 expression in L1J1 cells. Constitutive expression of Jagged-1 also caused a change in vessel structure in fat pads including less areas of branching than L1J1 fat pads. Thus, inappropriate expression of Jagged-1 impairs adipogenesis and subsequent adipose tissue development. Together this evidence provides

insight into one process that regulates adipose tissue development. A better understanding of such processes will help create therapies for people with disorders in adipose tissue development, such as obesity and Alagille syndrome.

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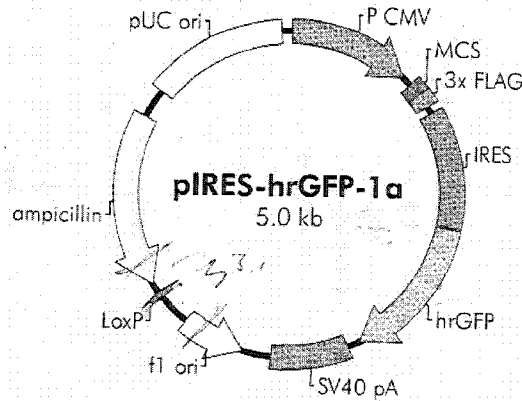
APPENDICES

APPENDIX A.

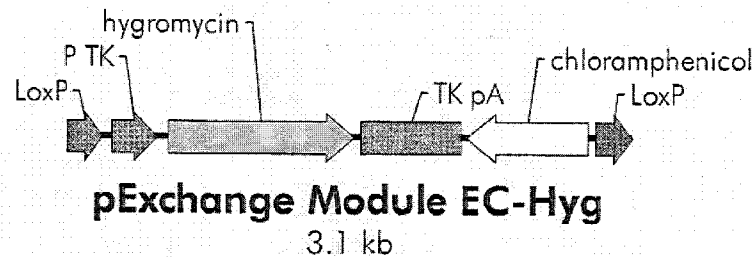
Generation of GFP Cell Lines

Following the manufacture's protocol, 0.5µl of pIRES-hrGFP-1a vector, 1µl pExchange Module EC-Hyg (100µg) and 1µl Cre Recombinase, 1µl 10 X Cre Recombinase Reaction Buffer, and 6.5µl ddH₂O were combined in an ependorf tube. Samples were heated for 30 minutes at 37°C followed by 20 minutes incubation at 65°C. 2.5µl of vector/ module suspension was mixed with 50µl of bacteria (DH52, sub-cloning efficiency, Invitrogen). The mixture was gently tapped and incubated on ice for 20 minutes. Samples were heat shocked in a water bath (42°C) for 1 minute and then placed back on ice for 10 minutes. 500µL of SOC media was added to each sample and samples were left to agitate for 1 hour at 37°C. Samples were plated on agar plates containing both chloramphenicol and ampicillin, and incubated at 37°C overnight. Individual colonies that took up the DNA were isolated the next day and screened for the presence of the PIREs-hrGFP-1 Hygro Plasmid.

A.A.



A.B



A.C

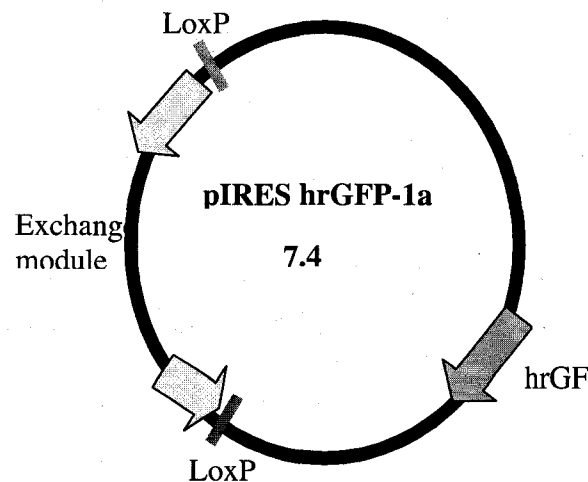


Figure A. 15. Our preadipocytes contained a hygromycin resistant and expressed GFP. The above images are graphic representations of the module and vector used for the generation of cell lines. A.A.) The above image is the vector which contained the GFP coding sequence. A.B.) The above diagram is a representation of the module which contained the hygromycin resistant gene that was cloned into the vector before transformation. A.C.) The above diagram a representation of the final product after the module was transformed into the vector.

GFP-Hygro myosin constructs were purified from these colonies using a plasmid isolation kit (MiniPrep, Qiagen), following the manufacturer's protocol. Briefly, bacteria pellets were re-suspended in 25 μ l Buffer P1 and transferred to a microcentrifuge tube. After 25 μ l of Buffer P2 was added to the sample, and tubes were inverted 5 times to gently mix. 35 μ l of Buffer N3 was added to each tube, and tubes were inverted. Samples were centrifuged for 10 minutes at 13,000 rpm, and resulting supernates were centrifuged in a QIAprep Spin Column for 30 seconds. A spin column was added to each tube to bind DNA in samples. Samples were washed in with 0.5ml Buffer PB and centrifuged for another 30 seconds. The flow through was discarded and the spin column was washed with 0.75ml Buffer PE and centrifuged for another 90 seconds. DNA was eluted into a clean tube with 50 μ l of Buffer EB (10mM TrisCl, pH8.5). Samples incubated for 1 minute followed by a centrifugation for 1 minute. A restriction digest was done on the samples to determine that samples contained the genes of interest. Enzymes; Kpn1 and Sca1 were used for the restriction digest. Along with samples, aliquots of GFP vector only were also cut with the enzymes to compare final products.

To make stable cell lines expressing GFP protein, L1 control and L1J1 cells were transfected with the vector pIRES-hrGFP-Hygro. Purified pIRES-hrGFP-1a was transfected into L1 and L1J1 cell culture. To begin, for each cell line mixtures of 750 μ l Optimem, and 30 μ l Optifect (Invitrogen) were prepared and incubated for 5 minutes. Next, 188 μ l (10 μ g/ μ l) of purified constructs was

added to the Optifect and Optimem mixture and incubated for 20 minutes. During this incubation period, L1 and L1J1 cell cultures with 70% confluency were washed with 1X PBS. 5ml of Quiescence media (DMEM with 0.5% (v/v) FBS, 1% (v/v) anti-biotic and anti-mitotic, and 0.8mg/ μ g Gentacin) was added to each culture. A mixture of Optifect, Optimem, and construct solution was then added to each culture dish. Cultures were placed back in the incubator and the media was changed after 48 to L1 media containing hygromycin (0.004% hygromycin).

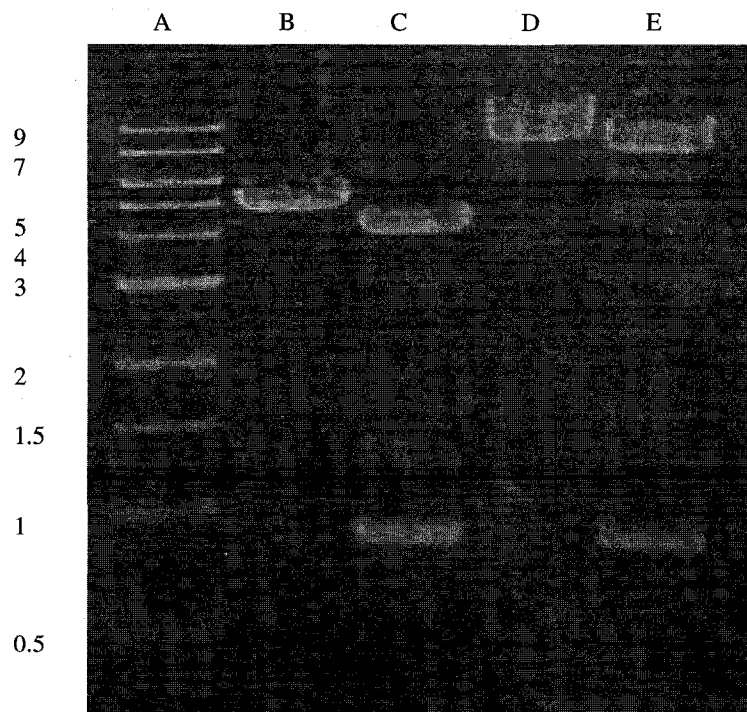


Figure A. 16. Restriction digest allows for visualization of plasmid DNA. Miniprep plasmid DNA was digested with Sca1 and Kpn1 to determine the identity of the plasmid DNA. Lane A is the DNA ladder, lane B is empty pIRES GFP-1a vector cut with Sca1, lane C is pIRES GFP-Hygro vector and cut with Sca1, lane D is GFP vector cut with Kpn1, and lane E is the GFP vector containing the hygromycin cut with Kpn1.

APPENDIX B.

TriReagent Protocol

Briefly, cells were scraped from culture-ware and lysed with 1ml of TriReagent by pipetting the cell suspension up and down. Each suspension was transferred to a microcentrifuge tube, which sat for five minutes at room temperature before being centrifuged at 12,000 x g for ten minutes at 4°C to pellet cellular debris and chromatin. The supernate was then transferred to a new microcentrifuge tube where 200µl of chloroform was added. Samples were briefly vortexed and allowed to remain at room temperature for ten to 15 minutes. Samples were then centrifuged for 15 minutes at 4°C to pellet cellular debris and insoluble protein. The resulting aqueous layer was transferred to an RNase free tube. 500µl of isopropanol was added to the samples and incubated at room temperature for five to ten minutes. To pellet RNA, samples were centrifuged at 12,000 x g for ten minutes at 4°C. Supernate was removed and pellets were washed with 1ml of 75% ethanol. Samples were centrifuged at 7,500 x g for five minutes at 4°C. The supernate was next removed and pellets were briefly allowed to dry. RNA pellets were re-suspended in 300µl of Diethylpyrocarbonate (DEPC) treated water (which inactivates Rnases) and stored at -80 °C.

APPENDIX C.

Qiagen mRNA Protocol

1mg of total RNA (adjusted with sterile water to give a total volume of 250µl) was added to an RNase free microcentrifuge tube along with 250µl of Buffer OBB (20mM TrisCl, pH 7.5, 1M NaCl, 2mM EDTA, 0.2% SDS), and 15µl of Oligotex Suspension (1mg/10ml Oligotex particles in 10mM TrisCl, pH 7.5, 500mM NaCl, 1mM EDTA, 0.1% SDS, 0.1% NaN₃). Samples were incubated at 70°C in a heating block, and incubated at room temperature for ten minutes. Tubes were centrifuged at 14,000 x g for five minutes to pellet Oligotex/mRNA. The supernate was removed and the pellet was re-suspended with 400µl of Buffer OW2 (10mM TrisCl, pH7.5, 150mM NaCl, 1mM EDTA). Samples were transferred to a new microcentrifuge tube with spin column and centrifuged for one minute at 14,000 x g. The spin column was transferred to a new microcentrifuge tube, and 400µL of Buffer OW2 was added to each column to wash the samples. Tubes were centrifuged for one minute at 14,000 x g. Spin columns were again transferred to a new microcentrifuge tube where 50µl (70°C) Buffer OEB (5mM TrisCl pH7.5) was heated to 70°C to elute mRNA from oligotex beads and added to the spin column. Samples were re-suspended and centrifuged at 14,000 x g for one minute. Spin columns were again transferred to a new microcentrifuge tube and another 50µl (70°C) of Buffer OEB was added to

the spin column. Samples were re-suspended and centrifuged at 14,000x g for one minute.

APPENDIX D.

PECAM-1 Staining Protocol

Sections for PECAM-1 staining were de-paraffinized and re-hydrated by sitting in ddH₂O for ten minutes, with the water being changed after five minutes. Antigen retrieval with Biogenex Trypsine pretreatment kit was used to incubate slides in 200-300mL of trypsin in a humid chamber for ten minutes at 37°C. Slides were then rinsed twice in PBS for five minutes and blocked for 30 minutes with TN blocking buffer. Next, slides were rinsed with TN buffer for five minutes and incubated in two to three drops of Avidin for 15 minutes at room temperature and then a repeated rinsed followed by incubation in two to three drops of Biotin. Slides were rinsed again and incubated for 30 minutes in primary antibodies (diluted in TN blocking buffer). After 30 minutes slides were washed in TN buffer three times for five minutes and incubated in SA-HRP (diluted to 1:100 TN blocking buffer) for 30 minutes at room temperature. Slides were rinsed three times and incubated in Biotinyl Tyramide Amplification (diluted at 1:50 in amplification buffer) for five minutes at room temperature. Slides were incubated again in SA-HRP for 30 minutes at room temperature, rinsed, and washed in PBS for five minutes. Slides were then incubated in DAB for five minutes, followed by a rinse in tap water. Slides were counterstained for two minutes in hematoxylin two and rinsed with tap water for two minutes. Sections were then incubated for one minute in Clarifier 2 and rinsed in tap water for two

minutes. Lastly, slides were incubated in Scott's Blueing Reagent for one minute, rinsed in tap water for one minute, dehydrated, and mounted with permount.

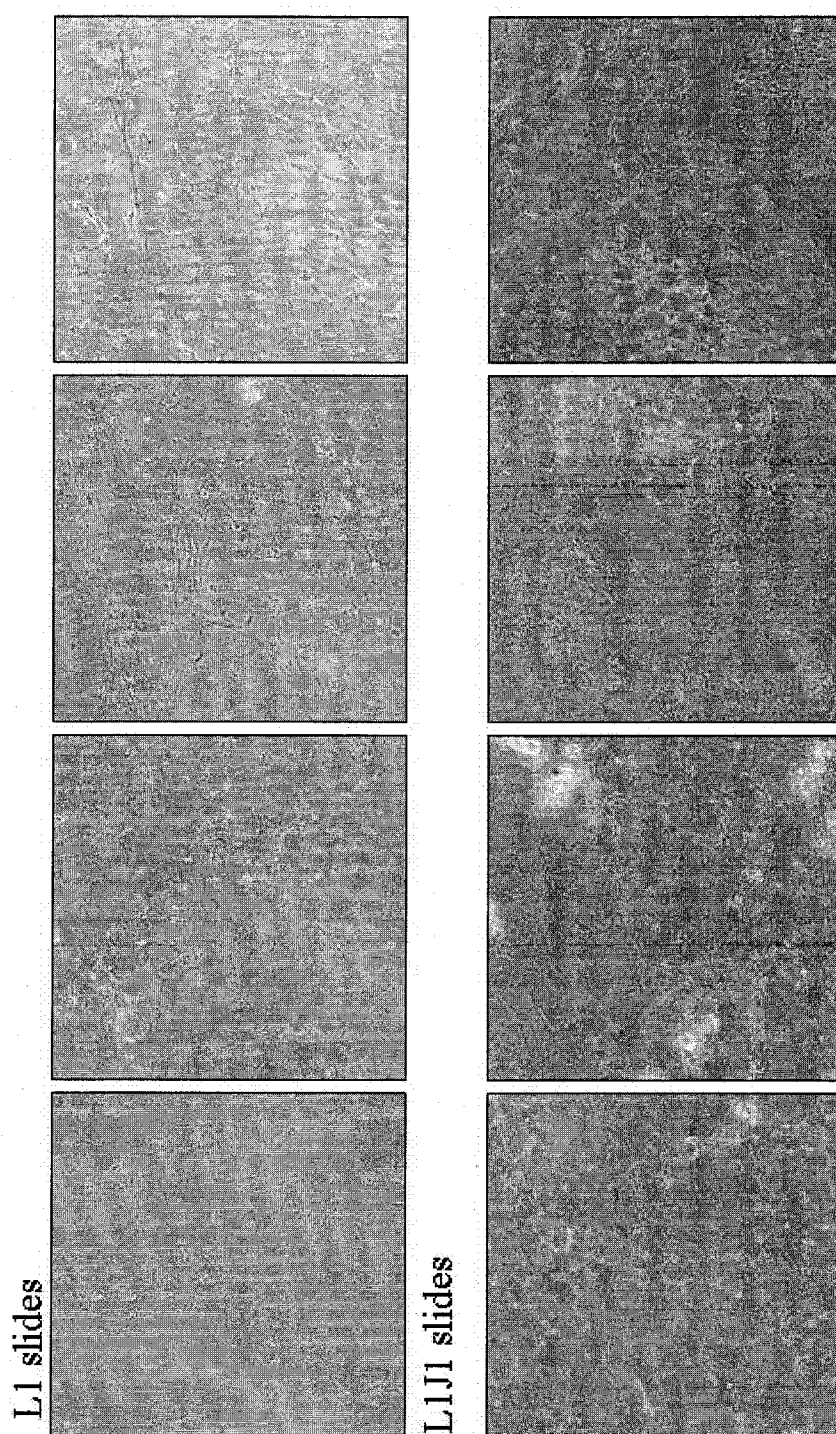


Figure A. 17. PECAM-1 staining of fat pad sections determined no difference in overall vessel area. Fat pad sections were stained against PECAM-1. Area on fat pad slides with positive PECAM-1 stain was quantified using Scion imaging. Total vessel area was compared for each fat pad type.

APPENDIX E.

IACUC Approval



UNIVERSITY of NEW HAMPSHIRE

February 27, 2006

Deena Smali
Biochemistry & Molecular Biology
Rudman Hall
Durham, NH 03824

IACUC #: 050205
Original Approval Date: 02/25/2005 **Next Review Date:** 02/25/2007
Review Level: C

Project: Role of Jagged1 in Adipogenesis and Adipocyte Function

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

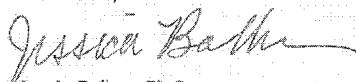
The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,


Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building,
51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564



UNIVERSITY of NEW HAMPSHIRE

February 27, 2006

Deena Small
Biochemistry & Molecular Biology
Rudman Hall
Durham, NH 03824

IACUC #: 040206
Original Approval Date: 02/25/2005 **Modification Approval Date:** 02/24/2006
Review Level: C

Project: Role of Jagged1 in Adipogenesis and Adipocyte Function

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the requested modification to the protocol for this study:

- Changes in animal numbers per year but not total number of animals

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair
air

cc: File

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51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564